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8

Development of an Ultrasensitive CRP Latex Agglutination Reagent by Using Amino Acid Spacers

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1. Introduction

William S. Tillett (1930) discovered that C-Reactive Protein (CRP) is an acute-phase serum protein that reacts with the *C* polysaccharide of *Pneumococcal cell wall*. CRP is present in human serum and its level increases during systemic inflammation. Therefore, CRP represents a sensitive marker of systemic inflammation and tissue damage; precise determination of CRP levels has proven to be useful in the screening of various diseases, monitoring treatment response, and detection of concomitant infections[1)].

In recent years, studies have indicated that arteriosclerosis may be due to vascular inflammation, and that CRP concentration in the blood correlates with the degree of functional disorder of the vascular endothelium. High CRP serum levels have been shown to be a risk factor for anginal damage or myocardial infarction-induced stroke[2-4)]. Therefore, a highly sensitive and accurate method of measuring CRP is clinically significant.

We conducted a sensitive quantification of CRP concentration using latex turbidimetric immunoassay (LTIA). This method measures turbidity changes in the latex immunoagglutination reaction using a latex reagent containing an antibody or antigen conjugated to latex particles[5-8)]. LTIA is a simpler, more rapid, and more highly sensitive assay of CRP compared with radioimmunoassay (RIA) and enzyme immunoassay (EIA). LTIA can measure CRP concentrations at levels between 10^{-10}–10^{-11} mol/l of serum, and can be performed using automated systems for high throughput immunoassay analysis.

In this study, we investigated a latex reagent containing an amino acid spacer to measure high-sensitivity CRP. We measured serum CRP levels in healthy individuals, people with liver disease, and diabetics. We report that this method is highly useful and clinically beneficial.

2. Materials and methods

2.1 CRP antigens

We used two kinds of whole CRP and five forms of CRP fragments. The first C-Reactive Protein High Control (human serum), termed Human CRP, was purchased from Dako

(Glostrup, Denmark) for use as an immunogen and standard. The second whole CRP protein, termed recombinant CRP (rCRP), was purchased from Oriental Yeast (Tokyo, Japan). This was used in the screening of hybridoma cells for the production of monoclonal antibodies. Five CRP fragments were prepared by the production of recombinant CRP fragments a method described below for epitope analysis.

2.2 Production of monoclonal antibodies (MoAbs)

Two 6 to 8-week-old BALB/c female mice (Oriental Yeast) were immunized biweekly with Human CRP. The CRP was injected in complete Freund's adjuvant (Sigma-Aldrich, St.Louis, MO) or incomplete Freund's adjuvant (Sigma-Aldrich) and sterile saline subcutaneously at multiple sites on the ventral area of the mouse. One week following the fifth booster immunization, tail blood was collected, and the ability of the antibody present in the serum to bind rCRP was determined by ELISA. Splenocytes were prepared 3 d after final immunization, and were used in cell fusion experiments.

The cell fusion experiments were described previously.[9] Briefly, immune spleen cells and a 1:5 ratio of P3U1 myeloma cells cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% v/v FetalClone Ⅱ (Thrmo Fisher Scientific, Waltham, MA) were fused using 50% w/v polyethylene Glycol 1500 in 75 mM Hepes (pH 8.0; Roche, Basel, Switzerland). The fused cells were then suspended in RPMI 1640 medium containing HAT Supplement (HAT medium, Invitrogen), and cultured in 96-well plates (10 cells/100 μl/well) under standard 5% CO_2 culture conditions at 37°C. After approximately 10 d in culture, the hybridoma supernatants were screened by ELISA. Antibody-secreting hybridomas were then expanded in HAT medium, cloned by limiting dilution, and cultured for a further 10 d. The hybridomas were then subjected to two further rounds of selection by ELISA screening and cloning by limiting dilution. Selected hybridomas were cultured in RPMI 1640 medium. The resulting supernatants were used in MoAb characterization. MoAbs were isotyped using the IsoStrip Mouse Monoclonal Antibody Isotyping kit (Roche). Antibodies were also prepared on a large scale, as ascitic fluid, by inoculating the relevant hybridoma cells into 10-week-old pristane-treated male BALB/c mice. The MoAbs included in the ascitic fluid were purified by ammonium sulfate precipitation at 50% and by protein A or protein G chromatography.

Care and treatment of the experimental animals conformed to the Nihon University guidelines for the ethical treatment of laboratory animals.

2.3 Detection of antibodies in serum and culture medium by enzyme-linked immunosorbent assay (ELISA)

rCRP diluted in 100 mM carbonate buffer (pH 9.6) was added to 96-well plates and incubated for 2 h at 25°C. After three washes with phosphate-buffered saline (PBS) containing 0.05% v/v Tween 20 (PBS-T), the wells were blocked for 1 h at 25°C with 5.0% w/v skim milk (Becton, Dickinson, Franklin Lakes, NJ) in PBS, and washed 3 times with PBS-T. The antibody diluted in mouse serum or hybridoma supernatant was appropriately diluted in PBS and added to the wells, and this was incubated for 1 h at room temperature. PBS in place of the antibody solution served as a negative control. After three washes in PBS-T, 100 μl of HRP-conjugated goat anti-mouse IgG secondary antibody (diluted 1:4,000

in phosphate buffer) was then added to the wells, and this was incubated for 1 h at room temperature. The wells were then washed with TBS-T, and peroxidase activity was measured colorimetrically using 100 μl of substrate solution containing 0.4% *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) in a solution of 0.1% citric buffer (pH 5.0), and H_2O_2 added at a final concentration of 0.015% immediately prior to use. Following incubation for 20 min at room temperature, the reaction was stopped by the addition of 2N H_2SO_4, and the absorbance at 492 nm was determined using a microplate reader (Spectra Max M2, Molecular Devices, Ontario, Canada).

2.4 Production of recombinant CRP fragments

2.4.1 Amplification of the CRP gene

Genomic DNA was extracted from whole blood using the GFX Genomic Blood DNA Purification kit (GE healthcare, Buckinghamshire, England). Using the extracted genomic DNA as template, seven forward primers containing the CACC sequence and one reverse primer (Table 1) were used to amplify five different *CRP* gene fragments, named CRP 01, CRP 02, CRP 03, CRP 04, CRP 05, CRP a F, CRP a F and CRP b R, CRP b R by polymerase chain reaction (PCR).

Primer	Sequence (5′→3′)	Tm (°C)	Length (bp)
CRP-01	5′-CACCATGTCGAGGAAGGCTTTTG-3′	70.5	612
CRP-02	5′-CACCTCGTATGCCACCAAGAGACA-3′	71.1	465
CRP-03	5′-CACCAGGGTGAGGAAGAGTCTGAAG-3′	70.1	276
CRP-04	5′-CACCGAAGGAAGCCAGTCCCT-3′	76.6	183
CRP-05	5′-CACCACCATCTATCTTGGCGGG-3′	71.3	105
CRP-R	5′-TCAGGGCCACAGCTGGGGTTT-3′	73.9	-
CRP-a-F	5′-CACCGACATGTCGAGGAAG-3′	64.5	300
CRP-a-R	5′-TCAGGACTCCCAGCTTGTACA-3′	64.7	-
CRP-b-F	5′-CACCGGGTACAGTATTTTC-3′	56.9	375
CRP-b-R	5′-TCAGTTAATCTCATCTGGTGA-3′	57.2	-

Table 1. Forward and Reverse primers for Recombinant CRP

2.4.2 Expression of recombinant CRP fragments

Amplified *CRP* gene fragments were separated by agarose gel electrophoresis containing 0.8 μg/ml of crystal violet. Separated DNA fragments were then extracted and purified using WizardSV GEL and PCR Clean-up System (Promega, Madison, WI).

pET100/D-TOPO vector (Invitrogen) was used to express the *CRP* fragments. pET100/D-TOPO vector and purified DNA were mixed, and the mixture incubated for 5 min at room temperature to complete ligation. *Escherichia coli* TOP10 competent Cells (Invitrogen) were then added to the mixture on ice, which was incubated for 15 min and transformed by the heat-shock treatment method for 30 s at 42°C. Transformed colonies were then identified by colony PCR. The recombinant plasmids were purified using the Wizard SV GEL and SV

Miniprep DNA Purification Systems (Promega), and were transformed into *E. coli* BL21 Star (DE3) (Invitrogen) by heat-shock treatment for 30 s at 42°C . S.O.C. medium (Super Optimal broth with Catabolite repression) containing 2% Tryptone, 0.5% Yeast Extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, and 20 mM glucose was then added, the mixture was incubated for 1 h, and the culture solution was transferred to Luria-Bertani broth containing ampicillin (100 μg/ml) and cultured for a further 20 h at 37°C. When an optical density (O.D, λ = 600 nm) of 0.7 was achieved, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. Following 1.5 h incubation, the cells were separated from the culture supernatant by centrifugation (5 min at 14,000 × *g*) and the *E. coli* pellet was lysed with lysis buffer containing 3 mM potassium dihydrogenphosphate, 47 mM dipotassium hydrogenphosphate, 0.4 M sodium chloride, 0.1 mM potassium chloride, 10% v/v glycerine, 0.5% v/v Triton X-100, and 1 mM Imidazole. The suspension was then subjected to three rounds of freeze-thawing, and was divided into soluble and insoluble fractions by centrifugation (5 min at 14,000 × *g*). The proteins in each fraction were then analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5 Epitope analysis by western blotting

Expressed CRP fragments were separated by SDS-PAGE on a 12.5% acrylamide gel and transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA) using the iBlot Gel Transfer Device and iBlot Transfer stacks mini (Invitrogen) at 20 V for 6 min. A PVDF membrane was then submerged in methanol and ultra pure water and sandwiched in the iBlot Transfer stack mini system. The membrane was blocked for 1 h at 25°C in 5.0% w/v skim milk in PBS-T and washed 3 times in PBS-T. The membranes were then incubated with 4 MoAbs, numbered 4, 5, 7, and 8, for 1 h at room temperature, followed by alkaline phosphatase (AP)-conjugated goat anti-mouse IgG for 1 h at room temperature. Finally, protein bands were visualized using a substrate solution prepared by diluting BCIP/NBT (Moss, Pasadena, MD) 1:10 in buffer (pH 9.8) containing 50 mM 2-amino-2-hydroxymethyl-1,3-propanediol, Tris, 50 mM sodium chloride, and 25 mM magnesium chloride hexahydrate.

2.6 Preparation of anti-CRP latex reagents using monoclonal antibodies (MoAbs)

The binding of antibody to latex particles was performed using the water-soluble carbodiimide (WSC) method[10] with 1-ethyl-3-(3-dimethyl-aminopropyl) (EDAC; Dojindo Laboratories, Kumamoto, Japan). In this method, carbodiimide activation of carboxylate groups on the surface of latex particles (under acidic conditions) produces an unstable reaction intermediate, O-acylisourea. O-acylisourea subsequently reacts with N-hydroxysuccinimide (NHS; Acros Organics, Antwerp, Belgium) to produce a stable NHS ester (Fig. 1).

Briefly, 0.1 ml of 10% polystyrene latex particles, 0.225 mm in diameter (G1225; JSR, Tokyo, Japan), was added to 1.9 ml of buffer A containing 0.05 mol/l 2-morpholinoethanesulfonic acid monohydrate (MES) buffer (pH 5.6). The mixture was then centrifuged at 22,600 × g for 20 min, and the precipitate was resuspended in 1.0 ml of buffer A. This suspension was then added to 2.0 ml of 20 mg/ml EDAC and 0.23 ml of 50 mg/ml NHS, both dissolved in buffer

A; the mixture was stirred for 30 min at room temperature to form the NHS ester. After production of the NHS ester, the suspension was washed twice with buffer A and the precipitate was resuspended in 1.0 ml of buffer A. The suspension was then added to 2.0 ml of 0.133 mmol/ml amino acid, and the mixture was stirred for 30 min at 37°C and centrifuged at 22,600 × g for 20 min. The precipitate was then washed with buffer A by centrifugation. Similarly, anti-C-reactive protein (anti-CRP) antibody (100 µg/ml in 1.0 ml of buffer A) (Immuno Probe, Saitama, Japan) was conjugated to the latex particles with an amino acid spacer. The latex-conjugated anti-CRP antibody was resuspended in 1.0 ml of buffer A, and the suspension was added to 1.0 ml of 1.0% w/v denatured bovine serum albumin (dnBSA). The suspension was then stirred for 30 min at 25°C and washed with buffer B, containing 0.1 mol/l Tris buffer (pH 8.2) by centrifugation at 22,600 × g for 20 min. The precipitate was then suspended in buffer B.

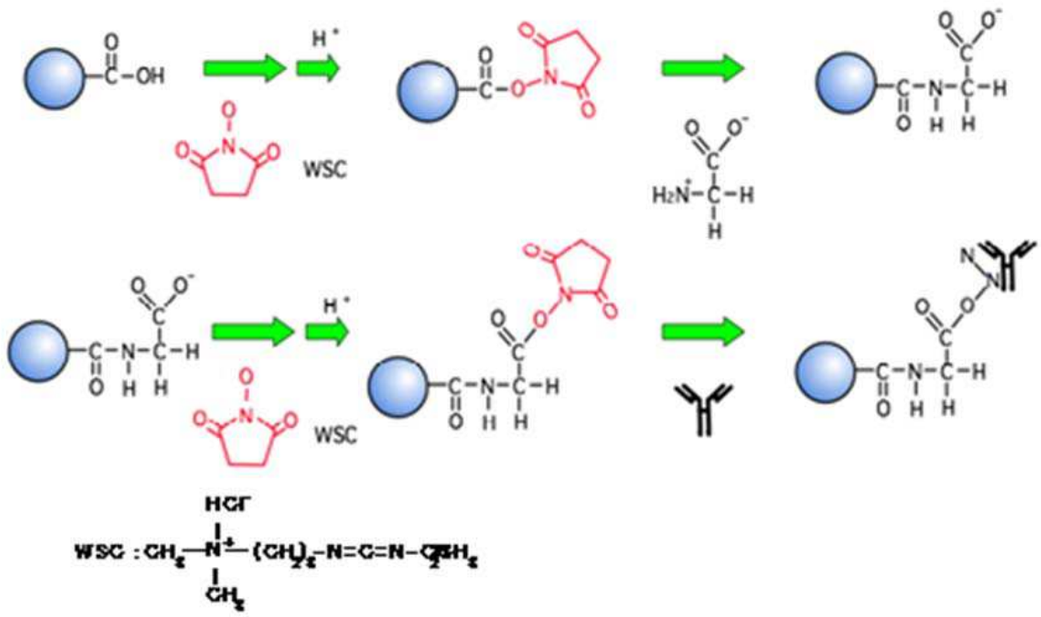


Fig. 1. Conjugation of antibody to Carboxyl Modified (CM) latex by carbodiimide.

2.7 Reaction conditions and evaluation of latex reagents using monoclonal antibodies (MoAbs) and polyclonal antibobies (PoAbs)

Latex agglutination due to the antibody-antigen reaction was measured by the automated latex photometric immunoassay system (LPIA-S500; Mitsubishi Kagaku Iatron, Tokyo, Japan).[6] To obtain calibration curves for CRP ranging from 0.5 to 200 ng/ml, 30 µl of standard CRP solution at various concentrations was diluted with 0.1 M Tris-BSA buffer (pH 8.2) containing 0.1 M Tris, 0.1% w/v EDTA•2Na, 0.8% w/v sodium chloride, 1% w/v BSA, and 0.1% w/v sodium azide following the manufacturer's directions. Thirty µl of sample and 50 µl of 0.1 M Tris-BSA buffer (pH 8.2) were then transferred to a plastic cuvette, and 40 µl of 0.25% latex reagent solution and 180 µl of 0.1 M Tris-BSA-PEG buffer (pH 8.2) containing 1% w/v PEG20000 were added. The rate of the latex agglutination was calculated by recording the absorbance at 800 nm at 12-s intervals, as reported previously.[6] Latex reagents prepared using the 4 MoAbs were evaluated for sensitivity, linearity, and

stability of latex agglutination. Specimens were properly diluted and measured for CRP concentration.

2.8 Anti-CRP polyclonal antibody and the F(ab')$_2$ fragment

Anti-CRP polyclonal antibody was produced by Immuno Probe (Saitama, Japan) The specific F(ab')$_2$ fragment was prepared by pepsin digestion, as previously described.[11)]

2.9 Clinical samples

We tested CRP levels in serum samples of 62 healthy individuals, 263 patients with liver disease, and 230 diabetic patients. Fifty diabetic patients had type 2 diabetes and were not under insulin treatment. All patients were examined in the Surugadai Nihon University Hospital, Tokyo, between April 2005 and March 2007. The study was approved in advance by the Ethics Committee of the hospital, and was conducted in accordance with the Helsinki Declaration. All study participants provided written informed consent prior to participation in this study.

3. Result and discussion

3.1 Characterization of monoclonal antibodies produced by established hybridomas

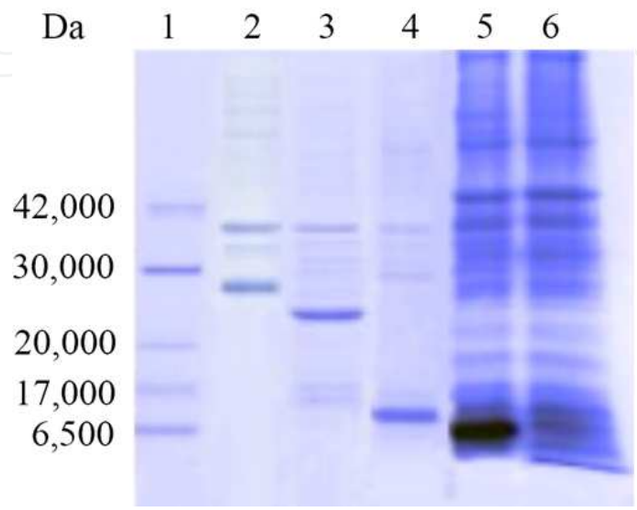
MoAbs produced from hybridoma cells were screened by ELISA, and seven MoAbs were subsequently established as stable hybridoma cells. Examination of MoAb reactivity revealed that hybridoma clone 5 had the highest sensitivity. Clonal sensitivity was ranked in the following order: clone 5 > clone 8 > clone 7 > clone 9 > clone 3 > clone 4 > clone 1. These isotypes and subclasses were then analyzed using the IsoStrip kit. The MoAb 1 produced from hybridoma clone 1 and clones 3, 5, 7, 8, and 9 were IgG1κ, while clone 4 was IgG2aκ. In addition, the specificity of the MoAbs was confirmed by western blotting using Human CRP. The MoAbs recognized a protein band at a molecular weight of approximately 11,500 Da. These results suggest that the MoAbs reacted specifically with CRP.

3.2 Epitope analysis

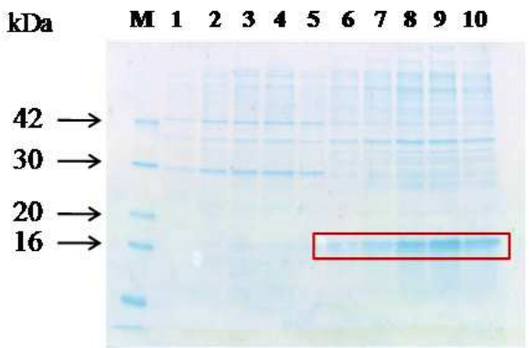
Protein epitopes are generally present in the areas of a protein that exhibit specific characteristics, including hydrophilicity,[12,13)] solvent accessibility,[14)] mobility,[15,16)] and the presence of protrusions.[13)] In addition, epitopes are commonly 5–10 amino acid residues containing loops and/or protruding regions.[17,18)] The hydrophilicity plot for the prediction of protrusions from the protein sequences can be measured by a simple method.[13)] Hydropathy analysis of CRP in this study was performed by the SOSUI system WWW-based tool (http://bp.nuap.nagoya-u.ac.jp/sosui/),[19)] since it was anticipated that hydrophilic epitopes would be present in CRP.

We mapped epitope locations using the seven CRP fragments. CRP-01 was 203 amino acid residues in length and was translated from the second exon (amino acid sequence positions 4 to 206); CRP-02 was 155 amino acid residues in length (amino acid sequence positions 52 to 206); CRP-03 was 91 amino acid residues in length (amino acid sequence positions 116 to 206); CRP-04 was 60 amino acid residues in length (amino acid sequence positions 147 to

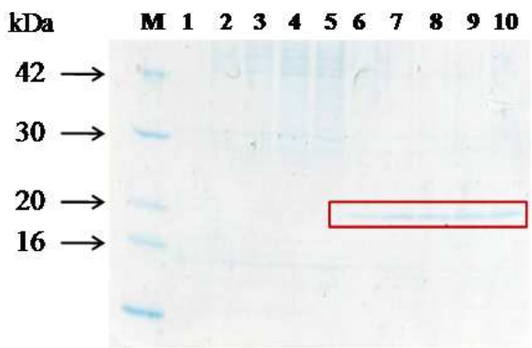
206); CRP-05 was 34 amino acid residues in length (amino acid sequence positions 173 to 206); CRP-a was 100 amino acid residues in length (amino acid sequence positions 4 to 103). and CRP-b was 125 amino acid residues in length (amino acid sequence positions 49 to 173). The seven recombinant CRP peptide fragments were then expressed in *E. coli* and their relative molecular weights were observed by SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 2). In each case, the relative molecular weight corresponded to the predicted size of the recombinant peptide fragments.



(A)



(B)



(C)

(A) Molecular weight marker (lane 1), CRP-01 of recombinant CRP fragment (lane 2), CRP-02 (lane 3), CRP-03 (lane 4), CRP-04 (lane 5), and CRP-05 (lane 6) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Recombinant CRP fragments for CRP-01, CRP-02, CRP-03, CRP-04, and CRP-05 correspond to molecular weights of 26.3, 19.9, 11,7, 6.8, and 4.0 kDa respectively. (B) Recombinant protein CRP-a, Lane 1-5: Add IPTG to culture pellet 0-5h, Lane 6-10: Culture pellet 0-5h. (C) Recombinant protein CRP-b, Lane 1-5: Add IPTG to culture pellet 0-5h, Lane 6-10: culture pellet 0-5h. M: Low range molecular weight marker (Wako Pure Chemical Industries)

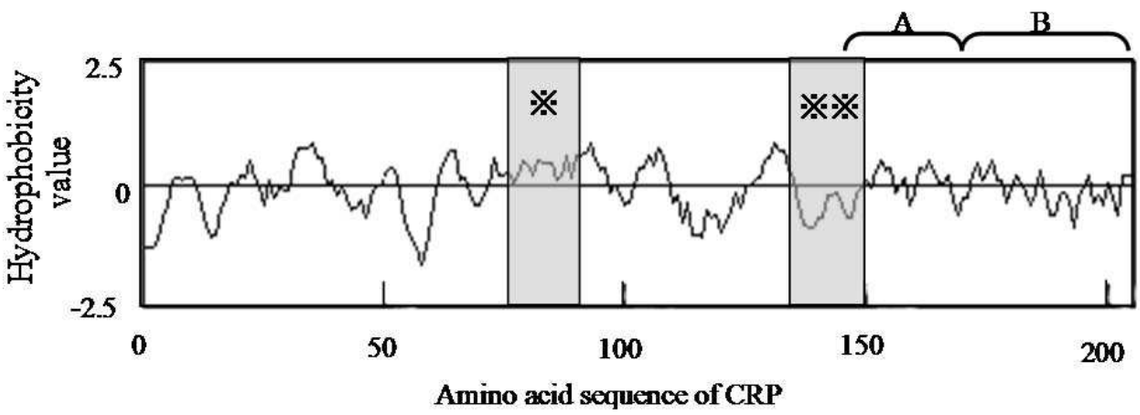
Fig. 2. SDS-PAGE Analysis of Recombinant CRP Fragments Expressed in *E. coli*.

The results of epitope analysis using Western blot are presented in Table 2. MoAbs 4, 7, and 8 were found to react with all fragments, while MoAb 5 reacted with CRP-01, CRP-02, CRP-

03, and CRP-04. This suggests that the C-terminal fragment (positions 173 to 206, Fig. 3B) contains the epitope for MoAbs 4, 7, and 8 while the fragment containing amino acid residues 147 to 172 (Fig. 3A) contains the epitope for MoAb 5. It is not a hydrophilicity epitope. However, these epitope regions include helix and loop structures, and there have been only a few reports of cross-reaction with these epitopes.[20,21] Hence, we hypothesized that these epitopes represent specific antigen sites.

Monoclonal antibody	Isotype	Reactivity with Recombinant CRP						
		CRP-01	CRP-02	CRP-03	CRP-04	CRP-05	CRP-a	CRP-b
No. 4	IgG2aκ	+	+	+	+	+	+	+
No. 5	IgG1κ	+	+	+	+	–	+	+
No. 7	IgG1κ	+	+	+	+	+	+	+
No. 8	IgG1κ	+	+	+	+	+	+	+

Table 2. Reactivity of the Anti-CRP Monoclonal Antibody with CRP Fragments by Western Blot Analysis



The C-terminal fragment from positions 173 to 206 (B) might contain the epitopes for MoAbs 4, 7, and 8, while the fragment spanning positions 147 to 172 (A) is thought to contain the epitope for MoAb 5.
※Cross-reactive site with heat shock protein 60.[20]
※※ Site similar to pentraxins in other species.[21]

Fig. 3. Hydrophobicity Plot of the CRP Amino Acid Sequence.

3.3 Reactivity of anti-CRP latex reagents using polyclonal antibody (PoAb)

3.3.1 Optimization of amino acid spacer concentration

First, bond concentrations of the amino acid spacers were examined. Preparation of latex reagent with amino acid spacers was synthesized using glycine at concentrations of 1.0–20 mg/ml, and the reagent produced binding antibody using latex synthesized at each glycine concentration. The examination of reactivity was performed to react CRP antigen with the binding antibody using the synthesized reagent. As seen in Fig. 3, the examination of reactivity improved as glycine concentrations increased. The optimum glycine concentration

was 10 mg/ml, and the excess glycine bond was decreased by the reactivity of latex reagent. Thus, latex responsiveness was improved by the combination of the amino acid and latex at a concentration of 0.26 mmol.

The result of each reagent using glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), and peptide synthesized by Fmoc solid phase (5 molecule bond of glycine: Gly5) are shown in Fig. 4. The rank order of reactivity was Gly5 > Gly > Ala > Leu > Val. Each amino acid produced a gentle sloping curve, and Gly5 produced a good reaction curve.

The accuracy of each amino acid is shown in Table 3. CV was about 0.5 or less and the stabilizing reagent was synthesized. Gly sensitivity is shown in Fig. 5. We demonstrated that it was possible to measure up to 500 ng/dl by calculating mean ± 3 SD values.

This study demonstrates that a latex reagent made by using 5 types of amino acid spacer molecules increased reactiveness by 40% as compared to using only 1 glycine molecule. It is possible that the long amino acid spacer reduces steric hindrance in the antigen-antibody reaction with the latex and thus reactiveness to the antibody is increased. Because the reactiveness is examined by using the amino acid, the position of the carboxyl group is the same as the amino group necessary for the peptide bond formation. Therefore, the length of the latex particle and antibody is constant. Because the alkyl group of the amino acid used is aliphatic, the hydrophobe of the amino acid, and the volume of the molecule are chiefly different (Table 4), the difference in reactiveness may be due to the side chain of the amino acid. As seen in Fig. 6, there was a 70% or greater increase in reactivity for all amino acids when the bonding amounts of the amino acid were compared, and a significant difference was not seen in the amount of antibody binding. Therefore, it is suggested that the reactiveness is due to the interaction of the aliphatic amino acid spacer with the hydrophobe.

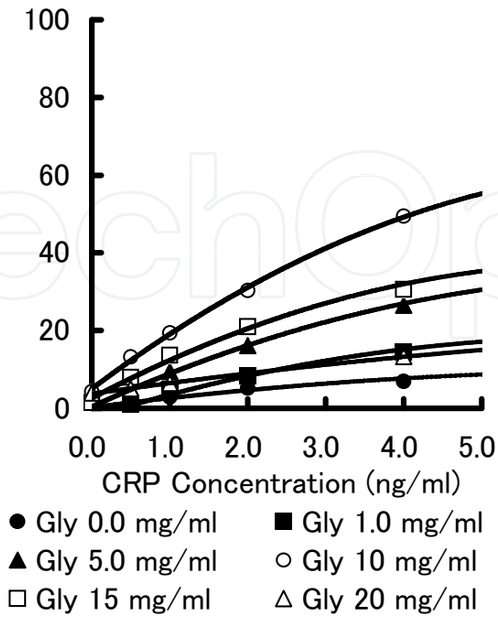


Fig. 4a. Comparison of reactivity in various concentration of glycine spacer

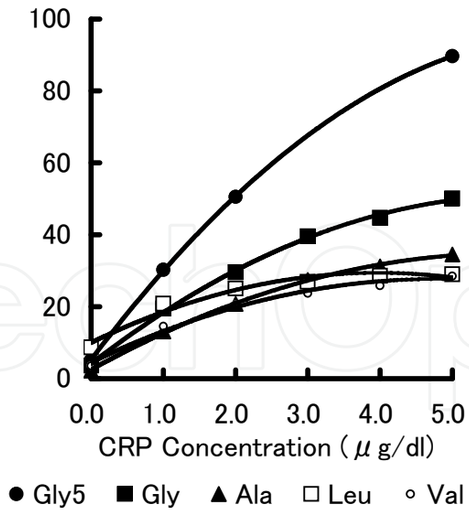


Fig. 4b. Comparison of reactivity in various types of amino acid spacers

Glycine						
Concentration (μg/dl)	0	1.0	2.0	3.0	4.0	5.0
Mean (absorbance)	0.058	0.288	0.430	0.481	0.542	0.583
SD	0.007	0.009	0.022	0.018	0.029	0.015
CV(%)	12.70	3.22	5.18	3.68	5.40	2.56
Alanine						
Concentration (μg/dl)	0	1.0	2.0	3.0	4.0	5.0
Mean (absorbance)	0.021	0.107	0.171	0.224	0.258	0.288
SD	0.010	0.012	0.021	0.030	0.033	0.041
CV(%)	49.44	11.24	12.26	13.53	12.92	14.06
Valine						
Concentration (μg/dl)	0	1.0	2.0	3.0	4.0	5.0
Mean (absorbance)	0.070	0.268	0.380	0.446	0.380	0.446
SD	0.006	0.016	0.022	0.026	0.022	0.026
CV(%)	8.74	5.89	5.85	5.81	5.85	5.81
Leucine						
Concentration (μg/dl)	0	1.0	2.0	3.0	4.0	5.0
Mean (absorbance)	0.191	0.389	0.515	0.550	0.604	0.603
SD	0.029	0.064	0.069	0.078	0.080	0.089
CV(%)	15.18	16.54	13.33	14.10	13.22	14.81
Gly5						
Concentration (μg/dl)	0	1.0	2.0	3.0	4.0	5.0
Mean (absorbance)	0.051	0.302	0.505	-	-	0.896
SD	0.05	0.081	0.083			0.093
CV(%)	15.54	10.95	7.32			7.96

Table 3. With in-run precision

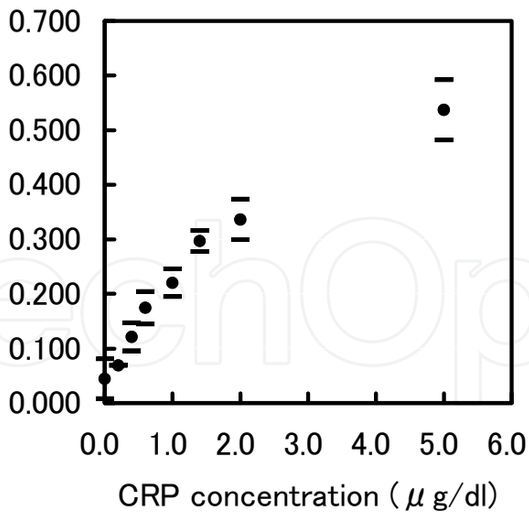


Fig. 5. Detection sensitivity

Amino acid	MW	p*I*	The solubility of amino acid in H_2O	
Glycine	75.07	5.97	14.18	(0°C)
			24.99	(25°C)
Alanine	89.09	6.00	12.73	(0°C)
			16.51	(25°C)
Valine	117.15	5.96	—	(0°C)
			8.34	(25°C)
Leucine	131.18	5.98	2.27	(0°C)
			2.19	(25°C)

Table 4. Amino acid spacers

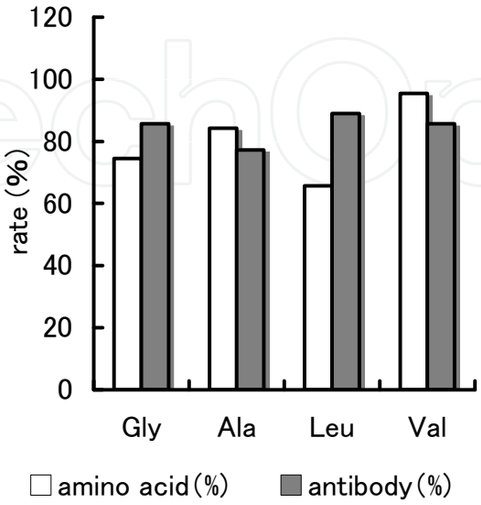


Fig. 6. Amount of conjugated amino acid and antibody

3.3.2 Immunoreactivity of prepared latex reagent

The latex reagent was produced by the method previously described using $F(ab')_2$ antibody, and the reactivity was compared with the conventional reagent. The $F(ab')_2$ reagent and conventional reagent were used to draw calibration curves from standard CRP samples and CRP levels derived from the 263 clinical samples from patients with liver disease. As seen in Fig. 7, the $F(ab')_2$ reagent correlated with a conventional reagent ($r^2 = 0.746$, $y = 0.4879$, and $x + 1.77$). Moreover, when CRP concentration was between 0.030 and 2.0 mg/dl, the correlation was $r^2 = 0.829$, $y = 0.909$, and $x + 0.6073$, indicating that there was a significant correlation at low concentrations. However, at high latex concentrations, this method was less sensitive.

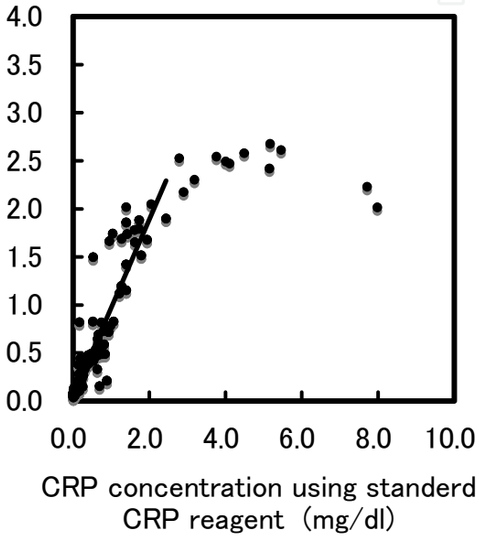
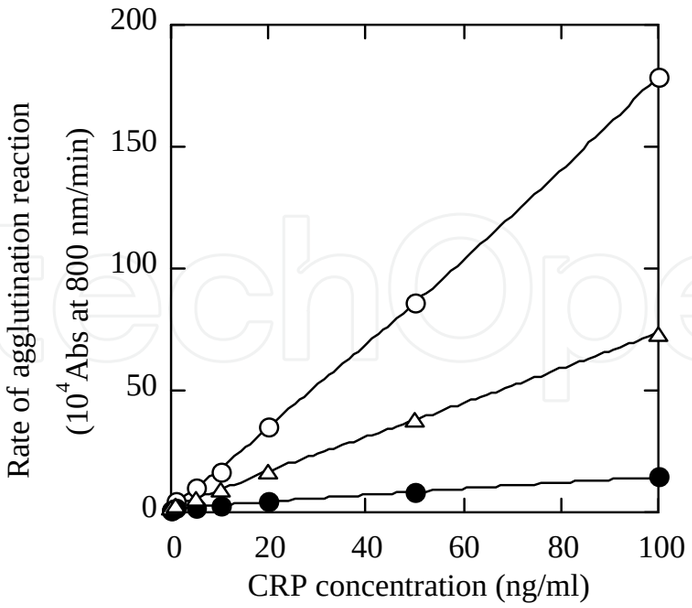


Fig. 7. Comparative study of CRP latex reagent

3.3.3 LTIA for titration of the MoAb and oligoclonal antibody

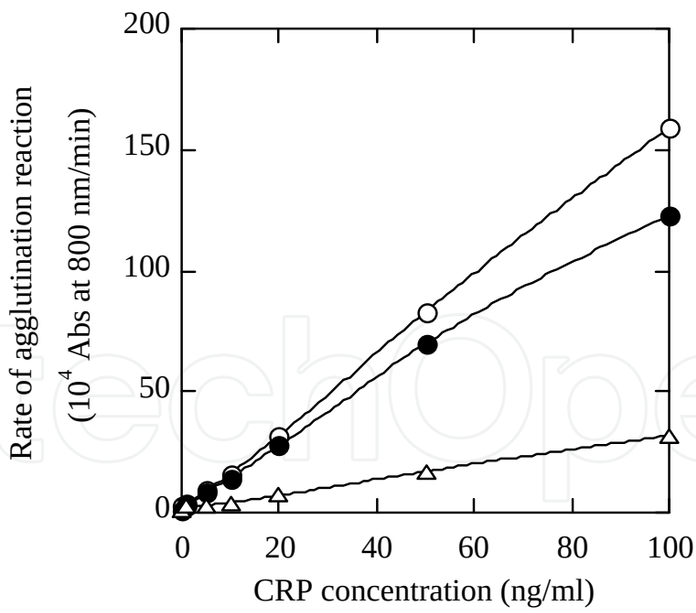
Three latex reagents with MoAb were used to quantify the CRP antigen by LTIA. The immunoreactivity curve of the latex agglutination rates using the various antibodies is shown in Fig. 8. We found that the reaction rates increased with the concentration of CRP antigen in an approximately linear fashion. The reactivity of these reagents was ranked in the following order: MoAb 5 > MoAb 8 > MoAb 7. This order of reactivity observed for the latex agglutination tests was found to be the same as that for the ELISA tests. The detection limit, which was calculated as the concentration equivalent to 3 standard deviations above the mean signal from 10 replicates of the zero standard, was calculated at 10 ng/ml.

Further, we prepared two types of latex reagent. First we mixed the two above-mentioned latex reagents together to form "mixed latex reagents," and combined these suspensions with CRP (Fig. 9). Then latex reagents were prepared using two MoAbs at a total antibody concentration of 100 μg/ml, and the latex reagent with two antibodies as the oligoclonal antibody were reacted with the CRP antigen (Fig. 10). The sensitivity of the latex reagents containing MoAb 5 was found to be the highest, and the latex reagents with the oligoclonal antibody were more sensitive than the mixed latex reagents. The lower limit of CRP antigen



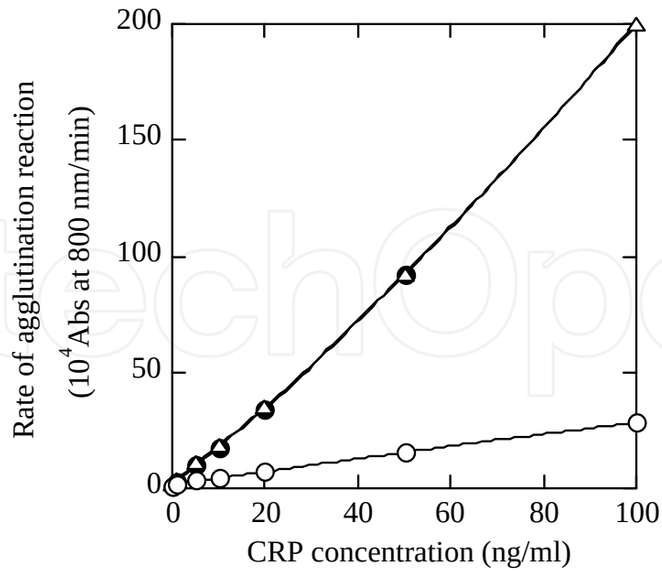
The latex reagent containing MoAb 5 (○) was found to have the highest activity, while the latex reagent containing MoAb 7 (●) was found to have the lowest activity. The latex reagent containing MoAb 8 (△) was found to have an intermediate level of activity.

Fig. 8. Comparison of Reactivity between Latex Reagents.



Three types of mixed latex reagents were prepared using MoAb 5 latex reagent and MoAb 7 latex reagent (○), MoAb 5 latex reagent and MoAb 8 latex reagent (●), and MoAb 7 latex reagent and MoAb 8 latex reagent (△). The mixed latex reagents containing MoAb 5 were found to have higher activity than the others.

Fig. 9. Comparison of Latex Reagent Reactivity Following Mixing of Two Types of Latex Reagents Containing Different MoAbs.



Three types of latex reagents with oligoclonal antibody were prepared with a combination of MoAb 5 and 7(●), MoAb 5 and 8(△), and MoAb 7 and 8(○). Two types of latex reagents sensitizing MoAb 5 were found to have higher sensitivity than the rest.

Fig. 10. Comparison of Reactivity Among Latex Reagents Containing Two Kinds of MoAbs.

detection, which was defined by the mean ± 3 SD method, was calculated as 10 ng/ml for the mixed latex reagents and 5 ng/ml for the latex reagent containing the oligoclonal antibody. When MoAbs for two different epitopes were used, the resulting latex reagent exhibited higher sensitivity than the MoAbs for two nearby epitopes. We suggest that latex reagents can be further increased in sensitivity through the use of MoAbs directed against remote epitopes.

3.4 Examination of clinical significances for quantification of CRP level in normal, hepatic disease, and diabetic subjects

The CRP level of sera in normal, hepatic disease, and diabetic subjects were measured by latex reagents prepared using glycine spacers (Fig. 11). Normal subjects (n = 62) showed a CRP average value of 0.03 mg/dl (maximum, 0.12 mg/dl; minimum, 0.01 mg/dl). These CRP levels in sera were 0.02 mg/dl lower than reference healthy individuals .

Patients with liver disease (n = 263) caused by hepatitis B and C had an average CRP level of 0.14 mg/dl (maximum, 2.7 mg/dl; minimum, 0.04 mg/dl) in sera.

Type 2 diabetes mellitus subjects (n = 230) had an average CRP level of 0.07 mg/dl (maximum, 1.9 mg/dl; minimum, 0.01 mg/dl).

Furthermore, CRP level in hepatitis B and C patients sera was compared with hepatic disease subjects classified as having hepatitis, hepatic cirrhosis, and hepatic cancer (Fig. 12). CRP levels tended to increase with the advancement of the disease.

This new method of assaying CRP, which uses a latex reagent made with a glycine spacer, shows that there is a tendency for CRP levels to increase with age. We were able to get CRP

readings at low concentrations (0.020 mg/dl) in a subset of healthy volunteers (n = 63) who were between 20–30 years of age. This method was also used to measure CRP levels in patients with liver disease, and the levels were correlated with disease advancement. In traditional CRP assays, small changes are not usually detected owing to viral infection. Moreover, because the liver produces CRP, the decrease of CRP production has been linked to hepatic infection. However, the change in such CRP level due to liver disease was confirmed by this new highly sensitive CRP density measurement. Our data indicate that CRP is produced by the inflammatory response of the liver in patients with hepatitis B and C.

We also measured CRP levels in patients with diabetes. Chronic high blood sugar in diabetic patients increases the risk of arteriosclerosis. Recent studies indicate that insulin-resistant diabetes is especially correlated with arteriosclerosis and that heightened level of CRP in type 2 diabetes may be a sensitive marker of arteriosclerosis.

This study indicates that CRP levels are not only elevated in people with particular infectious diseases, cardiac infarction, and arteriosclerosis, but also in the sera of people with liver disease and diabetes. These findings indicate that assaying CRP level will become more important for diagnosing multiple diseases and may be used to measure the effectiveness of therapies for patients with liver disease. Additionally, this newly developed method can detect even small changes in CRP levels and thus is much more sensitive than traditional methods.

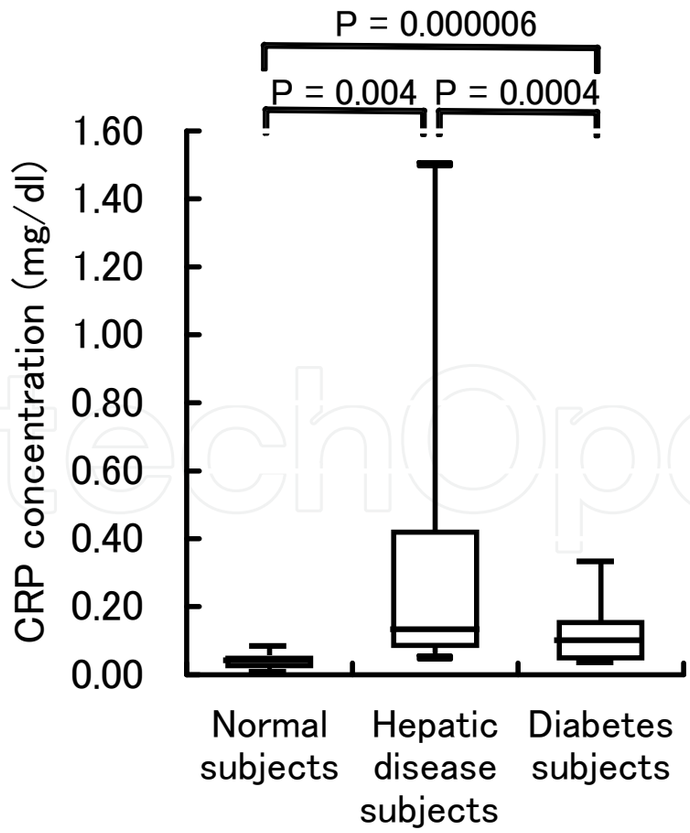


Fig. 11. Box and whisker plots in CRP measurement of normal subjects and patients

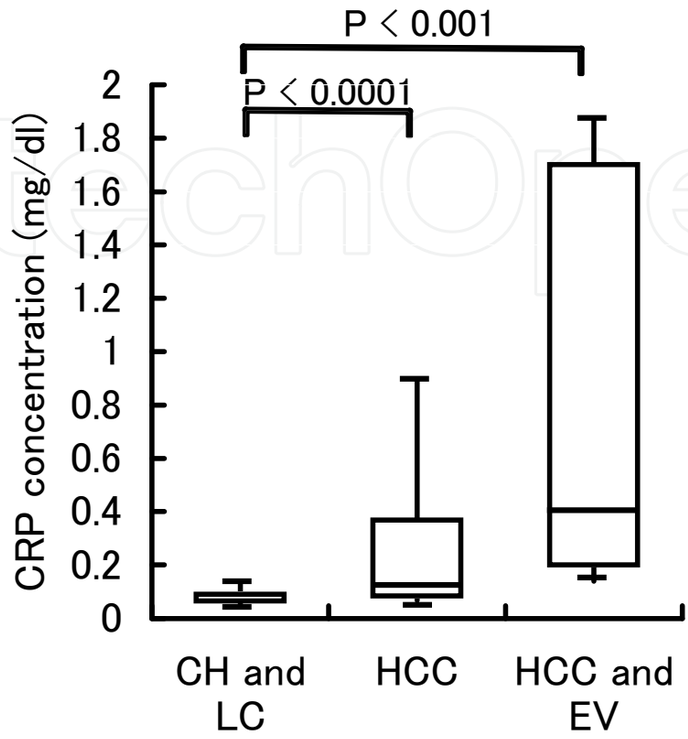


Fig. 12. Box and whisker plots in CRP measurement of various types of hepatic disease

4. Conclusion

We developed four novel MoAbs directed against CRP (MoAb 4, 5, 7, and 8), and classified these antibodies into two major groups. The epitopes for MoAbs 4, 7, and 8 were located between the amino acids residing at positions 173 to 206 of the CRP sequence. The epitope for MoAb 5 was located between the amino acids residing at positions 147 to 172 of the CRP sequence. These MoAbs were used in the preparation of latex reagents. The latex reagents constructed using these MoAbs were found to be highly sensitive. Moreover, the latex reagents, containing a cocktail of MoAbs specific for different epitopes, were also found to be highly sensitive. The lower limit of detection of CRP antigen, which was defined using the mean ± 3 SD method, was calculated to be 5 ng/ml for the latex reagents containing oligoclonal antibodies. Furthermore, the latex reagents that were prepared using three kinds of MoAbs reacted specifically with CRP-present patients with type-2 diabetes. In Fig. 12, the obtained CRP value, appear to be indicated the hepatitis disease situation with the extreme increased of CRP values in transition of disease phase from the point of these clinical evaluations. We concluded that high sensitive CRP measurement should be usable in the diagnosis of metabolic syndrome, such as diabetes and cardiac disease, early prediction of

infection disease, and clinical follow-up of infection of neonates that show low concentrations of CRP.

We suggest that latex reagents can be increased in sensitivity and specificity through the use of MoAbs directed against remote epitopes. The results from this study might also prove to be applicable to additional substances such as interleukin, *etc.*

This study presents a new CRP reagent with an amino acid spacer. In this method, the latex particles contain a glycine spacer, the anti-CRP antibody can be supported, and the orientation can be given to the antibody. As compared with other methods, this method allows for quick, efficient, and highly sensitive measurement of CRP levels even in low concentrations.

Additionally, we determined the serum CRP levels in a clinical sample of healthy subjects, people with liver disease, and diabetic subjects to be 0.1374 mg/dl, 0.0332 mg/dl, and 0.0704 mg/dl, respectively. There was a significant difference in the CRP levels between patients with type B and type C hepatitis. We also detected small differences in CRP levels between type 1 and 2 diabetic patients.

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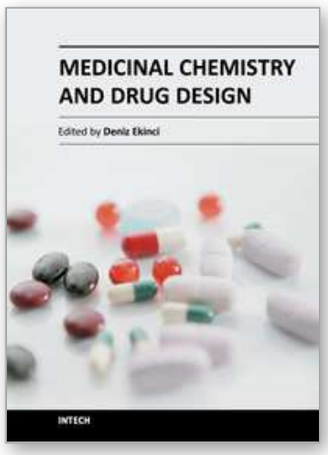

Medicinal Chemistry and Drug Design
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Over the recent years, medicinal chemistry has become responsible for explaining interactions of chemical molecules processes such that many scientists in the life sciences from agronomy to medicine are engaged in medicinal research. This book contains an overview focusing on the research area of enzyme inhibitors, molecular aspects of drug metabolism, organic synthesis, prodrug synthesis, in silico studies and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in medicinal chemistry and drug design. Particular emphasis is devoted to both theoretical and experimental aspect of modern drug design. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in associated areas. The textbook is written by international scientists with expertise in chemistry, protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the textbook will enhance the knowledge of scientists in the complexities of some medicinal approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of medicinal chemistry and drug design.

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