# Development of a Highly Sensitive Latex Reagent Directed against C-Reactive Protein (CRP) Using Epitope Analysis with Monoclonal Antibodies

Hideki KOHNO, Tomoe KOMORIYA and Masahiro OGAWA, (NIHON Univ.)

C-Reactive protein (CRP) is an acute-phase response protein that increases in amount during systemic inflammation. Following an initial acute-phase stimulus, the concentration of CRP increases by as much as 10,000 fold *via* a process termed *de novo* hepatic synthesis. This process is known to be regulated by pro-inflammatory cytokines, including interleukin-6.[1–3] Major CRP responses have been observed during periods of infection, sepsis, various autoimmunopathies, tissue necrosis, trauma, and neoplasia. The CRP concentration has also been found to correlate directly with disease progression through a range of proinflammatory properties.[1,4] Since CRP represents a sensitive marker of systemic inflammation and tissue damage, precise determination of CRP levels has proven useful in the screening of various diseases, monitoring treatment response, and the detection of intercurrent infections.[1]

Our laboratory determines the CRP concentration by 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 simple, rapid, and highly sensitive assay that can be performed using automated systems for high-throughput immunoassay analysis. In order to develop and engineer the latex reagent, an understanding of the physical properties of the latex particles, antibodies, and antigens is important, but there have been few studies on an antigen and its antibody during the latex agglutination reaction. LTIA for CRP measurement has been claimed to solve problems associated with CRP detection by providing higher sensitivity and specificity.

We have evaluated the CRP concentration by targeting the whole protein. In the present study, we determined CRP levels by targeting of small peptide fragments of the CRP epitope using monoclonal antibodies (MoAbs), and we evaluated the interaction of antigen-antibody reactive sites and latex agglutination to detect low concentrations of CRP.

**Materials and Methods**

*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.

*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.[9]

*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.[10] Obtained MoAbs were isotyped using the IsoStrip Mouse Monoclonal Antibody Isotyping kit (Roche). Antibodies were also prepared on a large scale, as ascitic fluid. The MoAbs included in the ascitic fluid

were purified by ammonium sulfate precipitation at 50% and by protein A or protein G chromatography.

*Production of recombinant CRP fragments*.

*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, five forward primers containing the CACC sequence and one reverse primer (Table 1) were used to amplify five different *CRP* gene fragments, named MK01, MK02, MK03, KM04, and MK05, by polymerase chain reaction (PCR).

*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. The PCR products were recombined into pET100/D-TOPO. The expression of recombinant CRP was then induced by isopropyl-β-D-thiogalactopyranoside. The proteins in each fraction were then analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS -PAGE).

*Epitope analysis by western blotting*. Expressed CRP fragments were separated by SDS-PAGE 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. The membranes were sensitized to anti-CRP MoAbs, and the 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.

*Preparation of anti-CRP latex reagents using monoclonal antibodies*. Binding of antibody to latex particles was done by the water-soluble carbodiimide (WSC) method[11] with 1-ethyl-3-(3-dimethyl-aminopropyl) (EDAC; Dojindo Laboratories, Kumamoto, Japan). By this method, carbodiimide activation of carboxylate groups on the surface of latex particles occurs only under acidic conditions and produces an unstable reaction intermediate named *O*-acylisourea. *O*-Acylisourea subsequently reacts with *N*-hydroxysuccinimide (NHS; Acros Organics, Antwerpen, Belgium) to produce a stable NHS ester.

Briefly, 0.1 ml of 10% polystyrene latex particles G1225 (0.225 μm in diameter; JSR, Tokyo, Japan) were added to 1.9 ml of buffer A containing 0.05 M MES (2-morpholinoethanesulfonic acid, monohydrate) buffer (pH 5.6). The mixture was then centrifuged at 22,600 × *g* for 20 min and the precipitate 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, and the mixture 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 L-arginine, 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, the IgG and F(ab')$_2$ fragments of the anti-CRP antibody (100 μg/ml in 1.0 ml of buffer A) were conjugated to latex particles with a L-arginine 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

**Table 1.** Forward and Reverse primers for Recombinant CRP

| Primer | Sequence (5′→3′) | Tm (℃) | Length (bp) |
|---|---|---|---|
| MK01 | 5′-CACCATGTCGAGGAAGGCTTTTG-3′ | 70.5 | 612 |
| MK02 | 5′-CACCTCGTATGCCACCAAGAGACA-3′ | 71.1 | 465 |
| MK03 | 5′-CACCAGGGTGAGGAAGAGTCTGAAG-3′ | 70.1 | 276 |
| MK04 | 5′-CACCGAAGGAAGCCAGTCCCT-3′ | 76.6 | 183 |
| MK05 | 5′-CACCACCATCTATCTTGGCGGG-3′ | 71.3 | 105 |
| MK R | 5′-TCAGGGCCACAGCTGGGGTTT-3′ | 73.9 | - |

denatured bovine serum albumin (dnBSA). The suspension was then stirred for 30 min at 25℃ and washed with buffer B, containing 0.1 M Tris buffer (pH 8.2) by centrifugation at 22,600 × *g* for 20 min. The precipitate was then suspended in buffer B.

*Determination of CRP concentration by latex photometric immunoassay.*

*Reaction conditions and evaluation of latex reagents*. Latex agglutination due to the antibody-antigen reaction was measured by the automated latex photometric immunoassay system (LPIA-S500; Mitsubishi Kagaku Iatron, Tokyo, Japan).[15] 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. Latex reagents prepared using the four MoAbs were evaluated for sensitivity, linearity, and stability of latex agglutination. Specimens were properly diluted and measured for CRP concentration.

*Clinical samples*. Fifty type-2 diabetic patients not receiving insulin treatment were recruited from 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.

## Results and Discussion

*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.

*Epitope analysis*

Protein epitopes are generally present in the areas of a protein that exhibit spcific 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

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

| Monoclonal antibody | Isotype | Reactivity with Recombinant CRP | | | | |
|---|---|---|---|---|---|---|
| | | MK01 | MK02 | MK03 | MK04 | MK05 |
| No. 4 | IgG2aκ | + | + | + | + | + |
| No. 5 | IgG1κ | + | + | + | + | − |
| No. 7 | IgG1κ | + | + | + | + | + |
| No. 8 | IgG1κ | + | + | + | + | + |

Symbols: +, reactive; −, not reactive

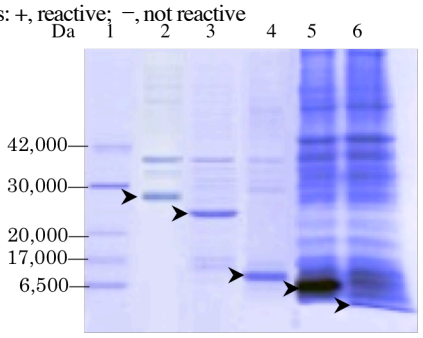


**Fig. 1.** SDS-PAGE Analysis of Recombinant CRP Fragments Expressed in *E. coli*.

Molecular weight marker (lane 1), MK01 of recombinant CRP fragment (lane 2), MK02 (lane 3), MK03 (lane 4), MK04 (lane 5), and MK05 (lane 6) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Recombinant CRP fragments for MK01, MK02, MK03, MK04, and MK05 correspond to molecular weights of 26.3, 19.9, 11,7, 6.8, and 4.0 kDa respectively.

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 five CRP fragments. MK01 was 203 amino acid residues in length and was translated from the second exon (amino acid sequence positions 4 to 206); MK02 was 155 amino acid residues in length (amino acid sequence positions 52 to 206); MK03 was 91 amino acid residues in length (amino acid sequence positions 116 to 206); MK04 was 60 amino acid residues in length (amino acid sequence positions 147 to 206); and MK05 was 34 amino acid residues in length (amino acid sequence positions 173 to 206). The five 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. 1). In each case, the relative molecular weight corresponded to the predicted size of the recombinant peptide fragments.

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 MK01, MK02, MK03, and MK04. This suggests that the C-terminal fragment (positions 173 to 206, Fig. 2B) contains the epitope for MoAbs 4, 7, and 8 while the fragment containing amino acid residues 147 to 172 (Fig. 2A) 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.

*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. 3. 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

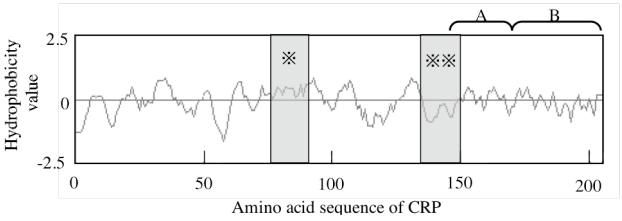


**Fig. 2.** Hydrophobicity Plot of the CRP Amino Acid Sequence.
The C-terminal fragment from positions 173 to 206 (B) might contain the epitopes for MoAbs 4, 7, and 8, while the fragment spanning positions 147 to 172 (A) is thought to contain the epitope for MoAb 5. ※ Cross-reactive site with heat shock protein 60.[20] ※※ Site similar to pentraxins in other species.[21]

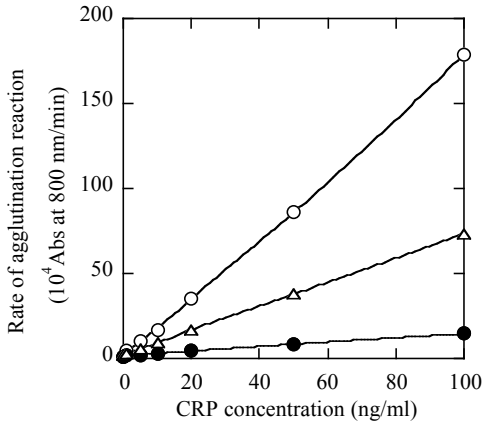


**Fig. 3.** Comparison of Reactivity between Latex Reagents.
The latex reagent containing MoAb 5 (○) was found to have the highest activity, while the latex reagent containing MoAb 7 (●) was found to have the lowest activity. The latex reagent containing MoAb 8 (△) was found to have an intermediate level of activity.

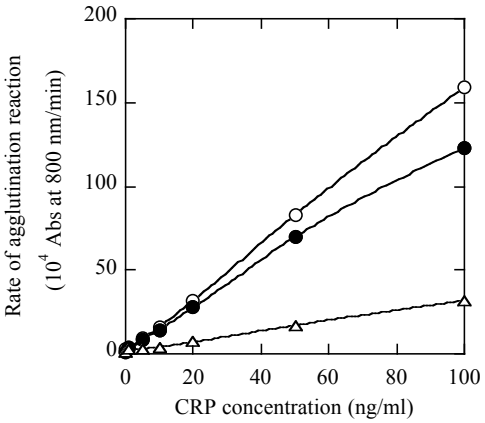


**Fig. 4.** Comparison of Latex Reagent Reactivity Following Mixing of Two Types of Latex Reagents Containing Different MoAbs.
Three types of mixed latex reagents were prepared using MoAb 5 latex reagent and MoAb 7 latex reagent (○), MoAb 5 latex reagent and MoAb 8 latex reagent (●), and MoAb 7 latex reagent and MoAb 8 latex reagent (△). The mixed latex reagents containing MoAb 5 were found to have higher activity than the others.

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. 4). 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. 5). 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 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.

*Measurement of CRP levels in clinical samples*

The CRP concentration in type-2 diabetes patients who were not receiving insulin treatment was determined by LTIA using LPIA ACE CRP-H II reagents and latex reagents containing IgG polyclonal antibody, $F(ab')_2$ polyclonal antibody, or MoAb 5, 7, or 8. The results revealed that the latex reagent combined with the polyclonal $F(ab')_2$ antibody exhibited a 1:1 correlation with the LPIA ACE CRP-H II reagent (Fig. 6). In the specimen assay, the measurement was undertaken using polyclonal $F(ab')_2$ reagent to prevent any non-specific activities of the reagent. This method of comparison demonstrated that the use of MoAb reagents yields results similar to those obtained using polyclonal antibody $F(ab')_2$ reagents. These findings indicate that we improved the sensitivity and specificity of CRP concentration assessment using specific MoAbs .

**Conclusion**

In the present study, we developed four novel

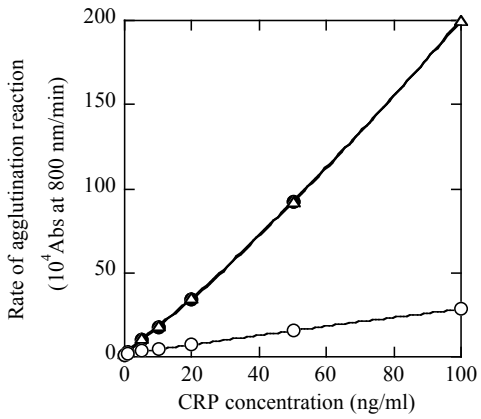


**Fig. 5.** Comparison of Reactivity Among Latex Reagents Containing Two Kinds of MoAbs.
Three types of latex reagents with oligoclonal antibody were prepared with a combination of MoAb 5 and 7(●), MoAb 5 and 8(△), and MoAb 7 and 8(○). Two types of latex reagents sensitizing MoAb 5 were found to have higher sensitivity than the rest.

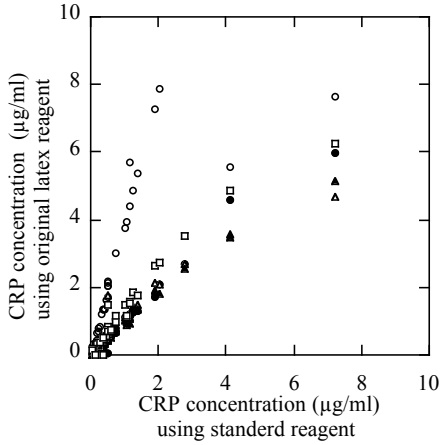


**Fig. 6.** Correlation between CRP Concentrations Obtained Using LPIA ACE CRP-H II Reagents and Those Obtained Using Prepared Latex Reagents Containing the IgG Polyclonal Antibody (○), $F(ab')_2$ Polyclonal Antibody (●), IgG MoAb 5 (△), IgG MoAb 7 (▲), or IgG MoAb 8 (□).

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. The CRP measurement should be useble 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*.

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