REVISTA ESPAÑOLA DE FISIOLOGIA, 44 (2), 163-168, 1988

Qualitative Screening of C-Reactive Protein by Latex in Microtiter Trays

J. M. Coll

Departamento de Virología
Instituto Nacional de Investigaciones Agrarias
c/. Embajadores, 68
28012 Madrid (Spain)

(Received on August 19, 1987)

J. M. COLL. *Qualitative Screening of C-Reactive Protein by Latex in Microtiter Trays.* Rev. esp. Fisiol., 44 (2), 163-168, 1988.

A microtiter-latex anti-C reactive protein method is described for screening large number of samples. The high percentage of false positives found with the slide-latex anti-C reactive protein method was reduced about 6 fold by the use of the described method. The use of microtiter trays, dilution of serum in one step and decreasing time of assay make this method simple, specific, rapid and easy to perform without sophisticated equipment.

Key words: C-Reactive protein, Latex, Microtiter.

C-reactive protein (CPR), a protein found in serum, increases from 5-10 mg/l in healthy adults to 500 mg/l in subjects with inflammation from multiple nonspecific diseases (11).

The following examples of the clinical usefulness of the estimation of CRP in serum have been reported: as one of the markers for detecting early colorectal carcinoma (8), assessing myocardial infarction (4), monitoring postoperative complications of patients (3), monitoring infections of the nervous system (10), detection of metastasis in human breast cancer (9), early diagnosis of neonatal septicemia (5), and differentiating between viral and bacteria infections (11).

The current use of slide latex C-reactive protein (CRP) takes 3 minutes per sample but it is inadequate for processing a large number of samples due to the critical timing of the process. In addition almost 40 % of the CRP positive samples when analyzed by either nephelometry or CRP ELISA were found to be negative (6).

This result could be due to: the irreproducibility of timing when a large number of samples is tested, influences of the variation of surface in the slide, evaporation, subjetivity, etc. By using microwells instead of slides, increasing the time of the reaction from 3 minutes to 60 minutes and increasing the serum dilution without introducing new steps, the facility of processing large number of samples and the specificity has been increased

Materials and Methods

Purification of human CRP. — The method of PONTET *et al.* (12) to purify CRP was used. In brief, human ascitic fluid was dialyzed against a solution of 20 mM Tris, 0.1 M NaCl, 10 mM $CaCl_2$ (pH 8) and passed through a 8 × 3 cm column of Sepharose 4B (Pharmacia, Uppsala, Sweden), connected to another column (7 × 1.2 cm) of agarose with immobilized 2-aminoethyl-dihydrogen phosphate (Pierce Chemicals, Rockford, IL, USA). Up to 500 ml of ascitic fluid could be processed each time. After washing the combined columns with the dialysis buffer, the 2 columns were eluted with 20 mM Tris, 0.1 mM NaCl, 20 mM sodium citrate, pH 8.

Two peaks were usually eluted from the aminoethyl-dihydrogen phosphate column; only the second peak gave a line by immunodiffusion against anti-CRP antisera (Biomerieux, Charbonnières les Bains, France). The fractions with CRP activity were pooled and dialyzed against the Ca^{2+} containing buffer described above. After discarding the precipitated material that appeared upon dialysis, the CRP was rechromatographed on the combined columns and separately eluted from the aminoethyl-dihydrogen phosphate column as above. The fractions with CRP activity were pooled and kept in the eluent at 4° C.

Immunodiffusion was carried out in the following medium: sodium borate 200 mM, NaCl 75 mM, $CaCl_2$ 2.5 mM, agar 10 g/l and merthiolate 100 mg/l (pH 8). CRP concentrations were calculated from absorbance at 280 nm, using an absorptivity (1 g/l) of 1.95 (13). Polyacrylamide gel electrophoresis with sodium dodecylsulfate and β-mercaptoethanol demonstrated only one band at 21 kDa.

Antisera against purified CRP. — To obtain antibodies against purified CRP, goats and rabbits were used. Two goats (30 kg weight) were i. m. injected daily with a total of 1 ml of purified CRP in 5 ml of 50 mM sodium phosphate, pH 7.4, mixed with 5 ml of Freund's adjuvant (Difco, Detroit, Mich. USA). Five rabbits (5 kg of weight) were i.s. injected daily with a total of 100 μg of the same fraction of CRP in 1.2 ml of 50 mM sodium phosphate, pH 7.4 with 1.2 ml of Freund's adjuvant. Complete Freund's was used in the first injections, 5 more were given with incomplete Freund's. Sixty days after the last injection, animals were bled by the jugular (goats) or by the ear vein (rabbits). Blood was allowed to clot and serum was obtained by centrifugation and frozen until use.

Preparation of $F(ab')_2$ fragment-coated latex. — IgG from antisera was precipitated by half saturation with ammonium sulfate and isolated by chromatography on diethylaminoethyl cellulose in 0.1 M Tris pH 8.5. After concentration IgG was digested with Pepsin (Sigma) at an enzyme substrate ratio of 1/50 in 0.1 M acetate pH 4.5. The F $(ab')_2$ fraction was then purified on a 2 × 80 cm column of ultrogel AcA 4.4 (L.K.B.) in 0.01 M phosphate pH 7.2. 0.1 M NaCl. The $F(ab')_2$ fraction was concentrated and dialyzed against 50 mM phosphate, 0.5 M NaCl, pH 7.2 (1, 2). About 600 μl of 0.8 μ particles of latex Estapor K 109 containing 10 % latex (Rhone-Poulenc) were washed with 10 ml of 0.05 M glycine, 0.03 M NaCl, pH 8.6, twice. They were resuspended in 20 ml of the same buffer and 150 μl of CRP antiserum added. The mixture was incubated at 37° C 3 h with agitation (7). In other experiments 1.5 mg of IgG or 2 mg of $F(ab')_2$ substituted the CRP antiserum. Then the latex suspension was washed in the same buffer once and finally resuspended in 5 ml of dilution buffer (Buffer A) 0.2 M sodium borate, 0.075 M NaCl, 2 mM $CaCl_2$, 1 % bovine serum albumin (BSA), 0.01 % merthiolate, 0.05 %

Tween 20, pH 8 and kept at 0° C. For use, this suspension was diluted 1/4 in dilution buffer.

Sera samples for correlation studies. — The blood samples from patients or from healthy controls (3-6 ml per sample) were allowed to clot and sera were separated by centrifugation and frozen at −20° C until use. The samples were to be assayed by four test methods: A) slide-latex, B) microtiter-latex, C) nephelometry and D) CRP-binding immuno enzyme assay (6). Standards were made by preparing dilutions of human ascitic fluid (800 μg/ml of CRP) in dilution buffer.

Slide-latex test. — The slide-latex test was carried out in glass slides. The anti-CRP latex spheres were from Lab. Diag. Morganville, N.J., USA. Fifty μl of serum were mixed with 50 μl of latex in a slide with a plastic stick. After 3 minutes agglutinated samples were classified as CRP positives and nonagglutinated samples were classified as CRP negatives.

Microtiter-latex test. — The microtiter-latex test was carried out in polyvinyl plates from Linbro (MRC-96, Handen, Conn. USA). Five microliters of serum were pippetted into each well, 50 μl of anti-CRP latex were added and all the samples agitated by moving the plates gentle and horizontally. They were allowed to stand at room temperature and read after 60 min against a black plastic. Homogenous latex corresponded to negative CRP samples whereas coarse pellets appeared in the positive CRP samples.

CRP-binding immuno enzyme assay. — The CRP-binding assay is a solid-phase enzymatic immunotest based on the use of PE-coated plates (Nunc, Kamstrup, Denmartk), Ca^{2+}−dependent binding, and development with anti-CRP antisera conjugated with peroxidase (EC 1.11.1.7). Microwell module F-16 medium binding capacity plates were coated with polylysine (Sigma) dried and then incubated overninght with *o*-phosphorylethanolamine (Sigma) and glutaraldehyde in 10 mM sodium phosphate, 150 mM CaCl, pH 7.4. After washing, the plates were kept at 4° C vacuum sealed until use. Under these conditions plates were stable for at least a year. Anti-human CRP goat serum was purified by an affinity column made by purify CRP and coupled to, peroxidase by the glutaraldehyde method. The assay procedure is as follows: the samples were diluted 961-fold in dilution buffer (buffer A) and 100 μl of this were incubated for 60 min at room temperature, then the plates were washed twice with the same buffer and incubated with 100 μl of anti-CRP-conjugated peroxidase for 30 min at room temperature. After washing five more times with the same buffer, 50 μl of 150 mM citrate buffer (pH 4.8) containing 3 mM H_2O_2 and 1 mg/ml of *o*-phenylenediamine, were added. The color development was stopped by adding H_2SO_4 4 M after 30 min. An EAR 400 FW spectrophotometer from SLT (Pasteur, Marnes-La-Coquette, France) at two wavelengths 492-620 nm was used to measure the absorbance on the wells. The absorbance at 620 nm is used to correct for individual-non significant differences between wells (6).

Nephelometry measurements. — The nephelometry measurements were made by rate nephelometry immunoassay (ICS; Beckman Instruments) with ICS reagents according to the manufacturer's instructions.

Results

Optimization. — For every latex preparation the amount of samples and latex had to be adjusted to define a 6 mg/l CRP cut off. The final concentration of

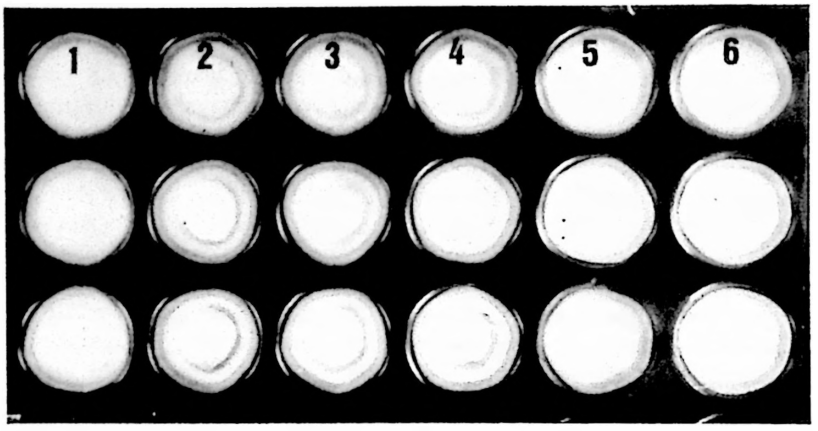

Fig. 1. *Settling patterns of CRP positive sera and CRP negative sera after reaction with anti-CRP latex.* Five microliters of serum were pippetted into each well of a polyvinyl V-shaped tray; after addition of 50 μl of anti-CRP latex, the tray was agitated horizontally for one minute and read after 60 minutes. Positive and negative controls were included. Positives were agglutinated (rows 2, 3 and 4) whereas negatives remained homogenous (rows 1, 5 and 6).

latex in the microtiter well must be 1 % for reaction to occur visibly in 30 to 60 minutes (figure 1). Lower final latex concentrations than these, results in longer times to obtain clear cut visual results to appear.

Time could be chosen for optimal differentiation between positive and negative serum samples. After 1 hour of settling the wells containing samples with CRP concentrations lower than 6 mg/l showed an hemogenous aspect whereas the wells containing samples with CRP concentrations higher than 6 mg/ml showed a visible pellet on the bottom due to the faster sedimentation of agglutinated particles. The negative reaction appears homogenous during several hours. The positive reaction appears as a coarse agglutination pelleted to the bottom of the well after 30 to 60 min. If more time is allowed for settling (about 1 day), the wells containing samples with low CRP content appeared with a very tiny and compact pellet whereas the wells containing samples with higher CRP concentration still showed a coarse pellet.

Several types of wells with different shapes and materials were tested and polyvinyl V shaped wells over black backgrounds gave the highest contrast and easily visible differences between positive and negative CRP containing samples.

The coating of latex with anti-CRP antiserum (0.75 μl/ml of 1 % latex), purified anti-CRP IgG (75 μg/ml of 1 % latex) or purified anti-CRP $F(ab')_2$ (100 μg/ml of 1 % latex) gave reagents that behave very similar in the agglutination reaction. By testing 50 serum samples with 32 % of positives the data obtained with latex coated with antiserum, IgG or $F(ab')_2$ agreed more than 94 %.

Comparison between slide-latex and microtiter-latex. — Eighty human serum samples classified as CRP positive by slide-latex were tested by microtiter-latex, nephelometry and CRP-binding, as described in Materials and Methods.

The true value of CRP concentrations was estimated by both nephelometry and CRP-binding, classifying the samples as negatives (CRP $\leq$ 6 mg/l) or positives (CRP $\geq$ 6 mg/l). The samples were also measured by CRP latex in microwells.

Table I. *Comparison of slide-latex (S-L) with microtiter-latex (M-L).*
Eighty human serum samples classified as CRP positives by the slide-latex were tested by microtiter-latex, nephelometry and CRP-binding. The true CRP values were considered the ones obtained by nephelometry and confirmed by CRP-binding.

	Percentage of the total number of samples	
	S-L	M-L
Double positives	61.2	43.8
Double negatives	—	32.5
False negatives (sensitivity)	—	17.5
False positives (specificity)	38.8	6.2

Positive and negative controls were included in each determination. Positives were agglutinated whereas negatives remained homogenous (figure 1). Samples were classified as double positives or negatives when both classifications coincided, as false negatives when a positive true value corresponded to a negative value in latex and as false positives when a negative true value corresponded to a positive value in latex.

By considering the correct CRP value of the samples the one obtained by nephelometry and confirmed by CRP-binding, the percentage of false positives could be reduced from 38.8 % in the slide-latex to 6.2 % in the microtiter-latex. The percentage of true values increased from 61.2 % in the slide-latex to 76.3 % in the microtiter-latex (table I). By the slide-latex method no negative CRP containing samples were detected whereas by the microtiter-latex 32.5 % of the samples were found as true negatives.

Discussion

The assay of human C-reactive protein by latex in microtiter trays is more specific than similar assays performed in slides (table I). Since in evaluating a test for serologic diagnosis the prime consideration is its efficiency and the classic definition of this is the percentage of samples correctly classified as positives and/or negatives, the microtiter-latex method was superior because correctly classified 76.3 % of the samples compared to the 61.2 % of the slide-latex method. This difference was mostly due to the low percentage of false positives in the microtiter-latex method (6.2 %) compared to the same percentage in the slide-latex method (38.8 %).

The relative increase in specificity of the microtiter-latex test could be due to the avoidance of the artefacts caused either by reading time differences sample to sample when a large number of samples are involved or by evaporation. One suggested source of interference in testing for CRP is the rheumatoid factor. This effect is minimized by about the 1 to 10 dilution factor of the sample (made in a single and simultaneous step with the addition of latex) compared to the 1:1 dilution factor in the slide-latex. This same dilution effect applies to possible interferences due to highly lipemic sera. Prozone phenomena could also diminished because of this higher latex to serum ratio.

The results of the microtiter-latex test by using latex coated with antiserum, purified IgG or purified $F(ab')_2$ were not different. This advantageous situation is probably due to the qualitative nature of the data, because $F(ab')_2$ fragments had to be used to reduce interference by serum constituents when latex is used for quantification by particle counting test (1, 2).

By using automatic pipettes, time to pippette 100 samples is 15 min and latex 4 min, which means 11.4 s per sample. Latex pippetting time could be lowered if multichannel pippettes are used. Working time required per sample is decreased mainly because no stick mixing of sample and latex is required. Mixing is done si-

multaneously to all wells. Waiting time for reaction to occur can be chosen between 1 hour (positives as coarse pellet, negatives no pellet) or 1 day (positives as coarse pellet, negatives tiny pellet) according to the convinience of the laboratory.

The microtiter-latex method described has, therefore, the following characteristics compared to the slide-latex method: simultaneous test of a maximum of 94 samples plus 2 controls (positive and negative), possible use of duplicates, is wanted; the polyvinyl plate can be cut with scissors adapting it to the number of samples; lower processing time per sample, higher time for reaction to occur; one same and simultaneous agitation to all the samples; clear cut difference between positive/negative result; the result is stable for several hours making timing not so critical; to assay for prozone, the negative samples could be reassay after 1 hour by adding another 50 μl of latex per well to the negative samples, agitating and waiting another hour; 1/10 dilution of the serum is made in the same step as latex addition; this step, possibly decreases unspecific agglutination due to high serum concentrations, rheumatoid factors and/or lipids; unspecific agglutinations are minimized because of the use of the same reaction volumes and surface; and evaporation artefacts found in the slide-latex test are avoided.

The use of microtiter trays, simple dilution of serum and increased time of individual reactions, make this method simple, specific, sensitive, rapid and easy to perform without the need for extensive personal training and sophisticated equipment. This method can be applied for screening large number of samples requiring only qualitative yes/no answers.

Acknowledgements

The author wishes to thank Dolores Navarrete for technical assistance and J. P. Albar for obtaining the $F(ab')_2$ fragments of anti-CRP IgG.

Resumen

Se describe un método de análisis de proteína C-reactiva para una gran número de muestras basado en el uso de látex en placas de microtitulación. El alto porcentaje de falsos positivos del método látex en porta-objetos se puede reducir 6 veces por el método descrito. El uso de placas de microtitulación, la dilución del suero en un solo paso y la disminución del tiempo de ensayo hacen este método simple, específico, rápido y fácil de ejecutar sin equipos complejos.

Palabras clave: Proteína C-reactiva, Latex, Microtitulación.

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