CLIN. CHEM. 33/12, 2185–2190 (1987)

Preliminary Clinical Studies of C-Reactive Protein Quantified by Enzyme-Linked Immunoassay

Jesus A. Martinez and Julio M. Coll[1]

We have used an enzyme-linked immunoassay technique to quantify human C-reactive protein (CRP). In this assay phosphorylethanolamine is covalently linked to polystyrene wells. Serum or plasma specimens are diluted 961-fold and assayed. After Ca^{2+}-dependent binding of CRP to the plates, the complex is reacted with peroxidase-labeled anti-CRP antibody. The response varies linearly with CRP concentrations between 10 and 160 mg/L; the detection limit is 0.34 ng per well. The results correlate well ($r \geq 0.90$) with those of rate nephelometry. This new method can be automated, is not subject to interferences or cross reactivity, is highly reproducible, has low background values, and can be carried out at room temperature.

Additional Keyphrases: *phosphorylethanolamine · peroxidase-labeled-antibody · calcium-dependent binding · rate nephelometry, latex agglutination, enzyme-linked immunoassay compared · ascitic fluid · reference interval · cutoff value · inflammation*

C-reactive protein (CRP), a protein found in serum, increases from about 5–10 mg/L in healthy adults to 500 mg/L in subjects with inflammation from multiple nonspecific diseases (*1*).[2]

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 (*2*), assessing myocardial infarction (*3*), monitoring postoperative complications of patients (*4*), monitoring infections of the nervous system (*5*), detecting metastasis in human breast cancer (*6*), making an early diagnosis of neonatal septicemia (*7*), and differentiating between viral and bacteria infections (*1*).

The CRP molecule, M_r about 107 500 (*8*), is made up of five identical nonglycosylated polypeptide monomers, noncovalently associated in a disc-like configuration with cyclic pentameric symmetry. This protein undergoes calcium-dependent binding to phosphorylamine compounds, e.g., phosphorylcholine (PC) and phosphorylethanolamine (PE). All of the biological and in vitro effects assigned to CRP seem to be initiated after it binds PC-containing substances (*9, 10*). Once complexed via its calcium-dependent binding site, CRP activates the classic complement pathway, starting with C_{1q}, and binds to lymphocytes bearing Fc receptors (*8*).

Current quantification methods (nephelometry, latex agglutination, radial immunodiffusion) have the general disadvantages accompanying agglutination and precipitation techniques (*1*).

Here we describe a new immunoenzymatic method for quantifying human CRP by binding to PE-coated solid-phase, soon to be commercially available as GENLIS® CRP assay (Invesgen, Madrid, Spain). This approach has several advantages over other methods:

- higher sensitivity and specificity
- easy automation for assaying a large number of samples (as with any other assay involving microtiter plates)
- no interference from other serum constituents: rheumatoid factor, serum amyloid protein (SAP), plasma, IgG, or hemoglobin (hemolysis)
- total 2-h incubation at room temperature, with few steps for reconstitution of reagents
- low background and wide range of assay linearity

Materials and Methods

Materials

Purification of CRP: We used the method of Pontet et al. (*9*) to purify CRP. In brief, the method is as follows. Human ascitic fluid was dialyzed against a solution containing, per liter, 20 mmol of Tris, 0.1 mol of NaCl, and 10 mmol of $CaCl_2$ (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 Chemical Co., Rockford, IL). Up to 500 mL of ascitic fluid could be processed each time. After washing the combined columns with the dialysis buffer, we separately eluted SAP and CRP from each of the two columns, using a solution containing 20 mmol of Tris, 0.1 mol of NaCl, and 20 mmol of citrate per liter (pH 8): "TSC eluent." Two peaks were usually eluted from the aminoethyl dihydrogen phosphate column; only the second peak reacted with anti-CRP antisera (Biomerieux, Charbonnières les Bains, France) by immunodiffusion. 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, we rechromatographed the CRP on the combined columns and separately eluted this protein from the aminoethyl dihydrogen phosphate column as above. The fractions with CRP activity were pooled and kept in the TSC eluent at 4 °C.

We used the following medium for immunodiffusion: borate 200 mmol/L, NaCl 75 mmol/L, $CaCl_2$ 2.5 mmol/L, agar 10 g/L, and thimerosal 100 mg/L (pH 8). We calculated CRP concentrations from absorbance at 280 nm, using an absorptivity (for 1 g/L solutions) of 1.95 (*10*). Polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate and β-mercaptoethanol demonstrated only one band at 21 kDa.

Pooled CRP-free human serum, and standards: Serum from at least 100 healthy persons was collected and pooled to a total volume of 500 mL. The sera were negative for both hepatitis B surface antigen ("Monolisa"; Pasteur, Marnes-La-Coquette, France) and HIV antibodies ("Elavia"; Pasteur). This pooled serum did not give a precipitation band by immunodiffusion against the anti-CRP antisera but, when measured by CRP binding to PE-coated wells, gave a positive result (quantified at 5 mg/L). We then dialyzed the

Departamento de Investigación Invesgen, Francisco Santos, 8, Madrid 28028, Spain.

[1] Address correspondence to this author.

[2] Nonstandard abbreviations: CRP, C-reactive protein; PC, phosphorylcholine; PE, phosphorylethanolamine; SAP, serum amyloid protein; TSC eluent, elution buffer containing Tris, NaCl, and citrate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunoassay.

Received December 23, 1986; accepted September 4, 1987.

serum against the dialysis buffer and passed the dialysate through a 3.5 × 2.6 cm column of agarose-immobilized 2-aminoethyl dihydrogen phosphate. Any CRP in the eluent, as measured by CRP binding to PE, was now below the detection limit of the assay (≤1 mg/L). We prepared standards by adding purified CRP to the dilution buffer and to diluted (961-fold) CRP-free pooled human serum.

Serum samples for correlation studies: The blood samples from patients or from healthy controls (3–6 mL each) were allowed to clot. The sera were separated by centrifugation and frozen at −20 °C until use. The samples were to be assayed by four test methods: (*a*) latex agglutination, (*b*) ELISA with pneumococcus cells, (*c*) rate nephelometry, and (*d*) the present ELISA method. All the samples in *a* were assayed with antibody-coated latex spheres (Laboratory Diagnostics, Morganville, NY); the samples classified as positive (CRP >6 mg/L) were used for the correlation studies. Samples in *b* were assayed by "Sopazyme," a commercial enzyme immunoassay (ITL, Brussels, Belgium) involving pneumococcus cells bound to microtiter plate wells, performed according to the manufacturer's instructions. Samples in *c* were assayed by a rate-nephelometric immunoassay (ICS; Beckman Instruments, Fullerton, CA) done with ICS reagents according to the manufacturer's instructions (*11*). The correlations between methods were determined by linear regression analysis by equal-weight least-squares fit (*12*).

Procedure

CRP-binding assay: The CRP-binding assay (method *d*) is a solid-phase enzymatic immunotest based on the use of PE-coated plates (Nunc, Kamstrup, Denmark), 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 10 μg/mL polylysine (Sigma Chemical Co., St. Louis, MO), dried, and then incubated overnight with 0.1 mol of *o*-phosphorylethanolamine (Sigma) per liter and 0.2 mol of glutaraldehyde per liter in phosphate-buffered saline (sodium phosphate 10 mmol, NaCl 150 mmol per liter, pH 7.4). After washing the plates we kept them vacuum sealed at 4 °C until use. Under these conditions, the plates were stable for at least a year. Goat anti-human CRP antiserum (Biomerieux) was purified on an affinity column made with purified CRP. The purified anti-CRP antibodies were coupled to peroxidase by the glutaraldehyde method (*12*).

The assay procedure is as follows: dilute the samples 961-fold in dilution buffer (per liter, 0.2 mol of sodium borate, 75 mmol of NaCl, 2 mmol of $CaCl_2$, 0.24 mmol of thimerosal, 10 g of bovine serum albumin, and 0.5 g of Tween 20, pH 7.5). Incubate 100 μL of this for 60 min at room temperature, then wash the plates twice with the same buffer and incubate with 100 μL of anti-CRP-conjugated peroxidase for 30 min at room temperature. Wash five more times with the same buffer. Then add 50 μL of 150 mmol/L citrate buffer (pH 4.8) containing 3 mmol of H_2O_2 and 1 g of *o*-phenylenediamine per liter. Stop the color development by adding H_2SO_4 (4 mol/L) after 30 min. We used an EAR 400 FW spectrophotometer (SLT Pasteur, Marnes-La-Coquette, France) to measure the absorbance of the wells at two wavelengths, 492 and 620 nm. We used the absorbance at 620 nm to correct for individual nonsignificant differences between wells.

Interference assays: We measured the analytical recovery of purified CRP added to sera containing potential interferents. Each sample was tested in duplicate by measuring the CRP binding to PE. Six or 12 μL of a 1 g/L solution of pure CRP was added to 100 μL of serum (see Table 3 below). Ten sera containing low concentrations of CRP and 19 containing medium concentrations were assayed before and after the addition of CRP, 60 mg/L, as were 10 different rheumatoid-factor-containing sera (as classified by a commercial latex test, "Reumagen AR"; Biokit, Barcelona, Spain). A serum containing a high concentration of lipids (triglycerides >1.3 g/L) was assayed before and after the addition of 125 mg of CRP per liter. We calculated the percentage recovery as [(final CRP minus initial CRP)/amount added] × 100.

Increasing concentrations of other potentially interfering substances were added to various human sera; we measured CRP concentrations before and after these additions.

The concentration of human IgG (Nordic, Tilburg, The Netherlands) was adjusted by measuring the absorbance at 280 nm and using $\epsilon = 1.4$. We added the human IgG to 961-fold dilutions of four different sera to give final concentrations equivalent to 9.6 and 96 g/L [normal concentration of IgG in human serum, 10–15 g/L (*13*)].

Human hemoglobin was isolated by repeated freeze–thaw treatment of packed human erythrocytes, followed by centrifugation. The concentration was adjusted by measuring the absorbance of heme at 403 nm and using a value of 80 for the millimolar absorptivity. We added human hemoglobin to the serum to give final concentrations of 0.7, 1.4, and 3.7 g/L [the maximum hemoglobin concentration in hemolysis is about 4 g/L (*14*)].

Human SAP was purified as follows: 1500 mL of human ascitic fluid was dialyzed and passed through a column of Sepharose 4B as described above for purifying CRP. After washing the column with the same buffer, we eluted SAP with the TSC eluent. After concentration by lyophilization, we passed the eluate through a 3 × 1.7 cm affinity column containing a monoclonal antibody against CRP (I5C4; unpublished) to remove any contaminating CRP. The eluted fraction did not give a positive immunodiffusion band against anti-CRP antisera from Biomerieux, and gave one 25-kDa band (in PAGE in 12–20% polyacrylamide gradient), just above the 21-kDa band for CRP. We added human SAP to sera to give final concentrations of 4, 13, and 22 mg/L [the usual SAP concentration in human sera is 33–43 mg/L (*15*)].

Heparin, trisodium citrate, and EDTA (obtained from Sigma, Probus, and Merck, respectively) were added to give the concentration ranges used for normal anticoagulant activity (*16*) to a serum containing 10 mg of CRP per liter. Heparin (167 kilo-int. units/g) was added to serum at final concentrations of 15 000 and 20 000 int. units/L. Trisodium citrate was added to give final concentrations of 1.6, 3.2, and 6.4 g/L, and EDTA to give final concentrations of 1 and 1.5 g/L.

For all the above interference measurements, we calculated the results as (amount found after addition of substance/initial amount) × 100.

Sera with differing CRP contents were measured in duplicate by the same analyst at 961- and 96-fold dilutions on eight separate days during two weeks. In other experiments we measured sera diluted 961- and 480-fold. The CRP concentrations were calculated from the standard curve, taking the dilution factor into account. Analytical recovery was calculated as follows: (average CRP concentration at 96- or 480-fold dilution/average CRP concentration at 961-fold dilution) × 100.

Results

Analytical Variables

Figure 1 illustrates a typical standard curve for CRP binding to solid-phase PE. No "hook" effect has been found, up to CRP concentrations of 1120 mg/L, but there is no further increase in response at concentrations greater than 160 mg/L (data not shown). The linearity of the assay response was studied with three different lots of reagents. In 11 assays we found that the assay response at 492 nm was linear between 0.3 and 1.8 absorbance units (corrected for absorbance at 620 nm), corresponding to 10–20 to 80–160 mg of CRP per liter. The correlation coefficient was 0.993 ±0.002 (mean ±SD) and the equation for the regression line was $y = (0.60 \pm 0.06) \ln x - (0.98 \pm 0.17)$, where y = corrected absorbance and x = concentration of CRP (mg/L). The corrected absorbance for a CRP concentration of 80 mg/L in 11 assays was 1.67 (CV = 10%).

The background-corrected absorbances obtained in 11 assays with dilution buffer, the CRP zero standard, and pooled human serum negative for CRP (diluted 961-fold) were 0.013 ±0.014, 0.035 ±0.019, and 0.032 ±0.016, respectively (mean ±SD). Assuming a normal distribution, we calculated the sensitivity and the detection limit of the assay. The sensitivity, defined as the lowest concentration that can be differentiated from the zero standard with 95% confidence, was 3.3 mg/L (0.34 ng per well). The detection limit, defined as the lowest concentration that can be differentiated from the results for the dilution buffer with 95% confidence, was 1 mg/L (0.1 ng per well).

Table 1 summarizes between- and within-run CVs. By assaying 170 samples in duplicate in four separate assays and dividing the samples into six concentration groups, we calculated that the CV varied between 3.7% and 8.3% for samples with CRP concentrations ranging from 0 to 160 mg/L.

The CRP-positive sera, as determined by latex assay (method *a*), had an average CRP concentration of 37.6 mg/L (n = 99) as quantified by CRP binding to PE (Table 2). The average CV, (ΣCV/99) × 100, was 5.14%. The average of the differences (d) between duplicates (range), expressed as a percentage of the mean $\bar{x}$, (Σd/99) × 100, was 9.8%.

Table 1. Within-Run and Between-Run Precision of CRP Binding to PE

	Within-run (n = 54 each)		Between-run (n = 8)[a]	
Sera[b]	Mean (SD) CRP concn, mg/L	CV, %	Mean (SD) CRP concn, mg/L	CV, %
Low	12.5 (0.5)	4	15.1 (1.7)	11.2
Medium	43.5 (2)	4.6	36.6 (3.6)	9.8
High	160.0 (12)	7.5	92.1 (8.6)	9.3

[a] Each sample was assayed in duplicate for eight different days within two weeks. The sera were kept at 4 °C, diluted 31-fold, throughout this period.

[b] CRP-negative sera gave nondetectable results (≤1 mg/L) in both within-run (n = 54) and between-run (n = 8) experiments.

Table 2. Precision Profile of CRP Binding to PE for Unknown Samples

Concn group, mg/L	n	Group mean (SD), mg/L	CV, %[a]
0–6	50	3.5 (1.5)	3.7
6–10	14	7.9 (0.9)	3.2
10–20	29	13.5 (2.5)	4.3
20–40	29	29.0 (6.0)	5.8
40–80	34	63.7 (10.0)	5.3
≥80	14	127.0 (54.0)	8.3

We analyzed 170 sera in duplicate in four separate assays and grouped the results by CRP concentration.

[a] Average CV of independent duplicates for each group of sera.

CRP Concentrations in Sera from Adults

Figure 2A shows the distribution of CRP values for sera from 36 healthy adults, assayed in a single run. The overall mean was 3.2 (SD 1.3) mg/L, but showed tailing off towards higher values. The mean for 14 men was 3.2 (SD 1.3) mg/L, 3.2 (SD 1.2) mg/L for 20 women. In two women, serum CRP

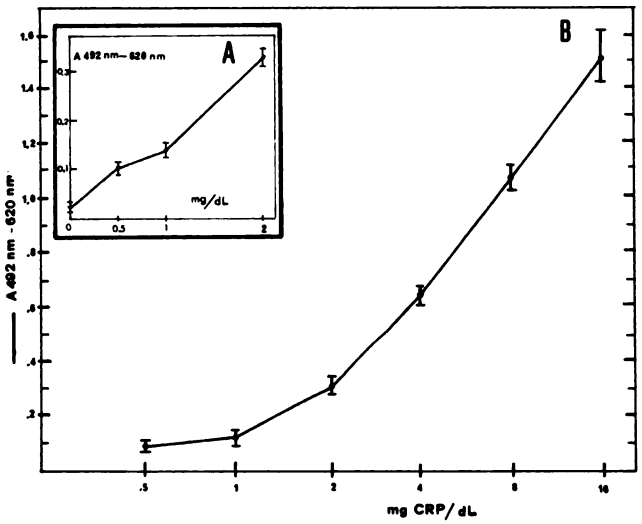

Fig. 1. Typical standard curve for CRP binding to solid-phase PE: (*A*) low concentrations plotted on a double linear scale; (*B*) results on semilogarithmic scale

All results are mean ± SD, n = 5. $A_{492} - A_{620}$ at 0 concentration of CRP in serum (background) = 0.024 (SD 0.01). The semilog curve is linear between 20 and 160 mg/L

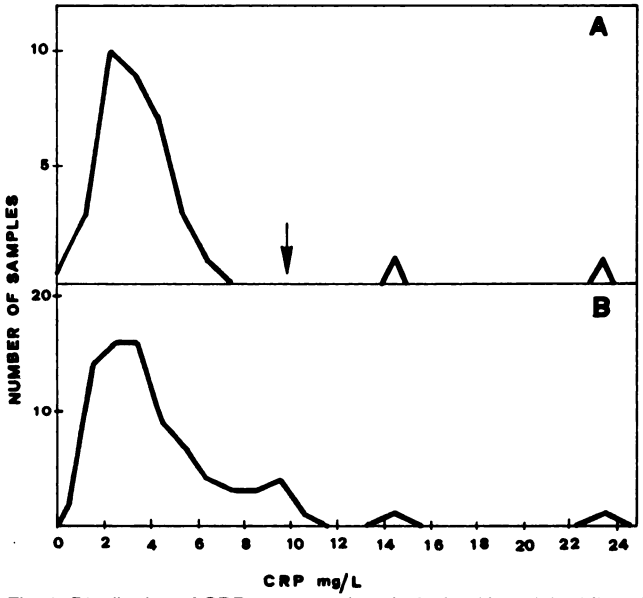

Fig. 2. Distribution of CRP concentrations in 36 healthy adults (*A*) and in 82 healthy or ill CRP-negative (by latex and nephelometry) donors (*B*)

Samples were assayed in two runs—one for *A* and one for *B*. The subjects' ages ranged from 20 to 60 years. The samples are distributed in 1 mg/L classes, with 0–0.9 mg/L being the lowest. The *arrow* in *A* indicates the "cutoff" point (see text)

exceeded 10 mg/L (14 and 23 mg/L). Pooling these results with those for 46 CRP-negative samples from ill patients affected the average value: 3.9 (SD 3.3) mg/L (Figure 2*B*). In another experiment (data not shown) the distribution of CRP values for sera from 38 healthy adults showed a mean of 3 (SD 3.6) mg/L, tailing towards higher values. For 19 men the mean was 2.3 (SD 2) mg/L, 3.7 (SD 4.7) for 19 women. Sera from two women and one man had CRP >10 mg/L (10 and 20 mg/L for the women and 10 mg/L for the man).

The normal CRP concentration in adults' sera can also be measured by pooling a great number of sera from healthy adults and measuring the resulting CRP concentration. For a 500-mL specimen of serum obtained by pooling 3–5 mL of individual samples collected over a three-week period, the mean CRP content was 5.6 (SD 1) mg/L (n = 6).

Results of some clinical studies with sera from patients with an increased CRP content are shown in Figure 3. CRP concentrations of 20 to 80 mg/L were found in patients with various diagnosed diseases. Most healthy adults had CRP concentrations ≤6 mg/L.

Analytical Recovery Studies and Possible Interferences

Table 3 summarizes the recovery of purified CRP added to various sera.

Measuring 10 samples with a positive rheumatoid factor reaction to latex, with and without added CRP, showed a correlation between the expected (y) and found (x) values of $r = 0.953$ ($y = 1.09x - 6.1$ mg/L).

The possible interferences due to normal serum constituents were studied by assaying CRP at serum dilutions of 96-, 480-, and 961-fold (Table 3, *h*). For example, the amount of CRP calculated for 96-fold diluted samples with an average CRP concentration of 7 mg/L was 68% of the amount calculated for 961-fold dilutions.

The concentration of Ca^{2+} needed to get half the maximum reaction was about 0.56 mmol/L. The addition of phosphorylethanolamine (0.1 mmol/L) during the first incubation step inhibits the final reaction by 50% (data not shown). Treating purified CRP with proteinase K, pepsin, or subtilisin (100 μg of CRP with 10 μg of enzyme) at 37 °C for 12 h completely digested the CRP, as evaluated by PAGE, and gave small background values when assayed by assay *d* (data not shown). The use of plastic tubes (polystyrene) or glass tubes for the dilution of the samples did not interfere in the assay (data not shown). CRP in human ascitic fluid, measured by binding to PE before and after purification on the 2-aminoethyl dihydrogen phosphate column (see *Materials and Methods*), was 60 and ≤3 mg/L, respectively.

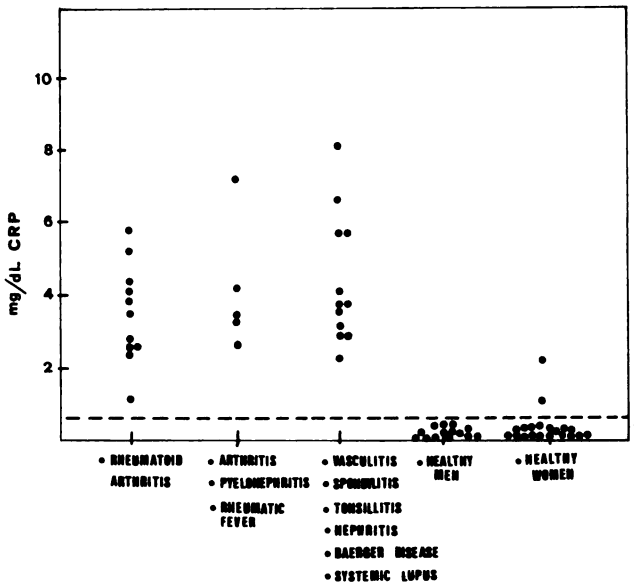

Fig. 3. CRP concentrations in serum samples from patients with various diseases as well as from healthy men and women

We assayed 28 serum samples from hospitalized patients and 34 serum samples were obtained from healthy donors (14 men and 20 women). The *dotted line* marks the boundary between insignificant (≤6 mg/L) and moderate increases of CRP concentration. The diagnosis of each patient at the time of blood collection was established retrospectively by the physician without knowledge of the serum CRP results. The diagnosis of the disease, serum collection, and assays were all performed in La Paz Hospital (Madrid)

Correlation with Commercially Available Assays

To study the correlations with other available assays, we assayed sera classified as positive by latex (method *a*), ELISA (pneumococcus) (*b*), nephelometry (*c*), and the present assay (*d*).

Figure 4 shows the distribution profiles of CRP values of 99 sera as determined by nephelometry and by CRP binding to PE. Table 4 summarizes the correlations between nephelometry or pneumococcus-ELISA with CRP binding to PE. In all, CRP from a total of 148 different sera have been quantified in five different assays by two different laboratories. In all the assays the correlation coefficient between nephelometry and CRP binding to PE was ≥0.9, and slopes were between 0.87 to 1.3. The coefficient of correlation with the pneumococcus-ELISA was slightly less: 0.89, with a slope of 1.14.

Ninety-nine sera with an average CRP content of 27.9 (SD 37) mg/L by nephelometry contained 37.6 (SD 50) mg/L when analyzed by CRP binding to PE. In other assays, 41 sera with an average CRP content of 23.5 (SD 34) mg/L by nephelometry contained 37.2 (SD 37.3) mg/L by CRP binding to PE. Assay of purified CRP (concentration adjusted by $\epsilon = 1.95$ and purity checked by PAGE) added to human CRP-negative pooled sera to give a final CRP concentration of 40 mg/L gave 40.25 (SD 3.8) mg/L (n = 2) by CRP binding to PE.

Sensitivity and specificity were calculated by assuming a cutoff value of 6 mg/L such as the one used in the commercially available latex test (assay *a*). Results are summarized in Table 5.

Discussion

The CRP binding to solid-phase PE provides an alternative for quantitative assessment of human CRP that does not involve precipitation, agglutination, or radioactivity.

The present method (Figure 1) has a background absorbance ≤0.04 *A*, compared with 0.2 *A* for method *b*, and a maximum absorbance of 1.6 *A*, compared with 0.8 *A* for method *b* (data for method *b* not shown). Both effects increase the measurable range of CRP concentration.

The sensitivity of this method for CRP was 0.34 ng per well, compared with similar values for method *b* (not shown) and detection limits of 6 mg/L for method *c* (*11*, *18*) and method *a* (data not shown).

The linear range extended from 10 to 160 mg/L (Figure 1), compared with 30–140 mg/L for method *b* (not shown) and 6–120 mg/L for method *c* (*11*) or 5–80 mg/L for another method *c*. The standard curve for the present method was within 10% (CV) as used with three different lots of reagents.

The within-run CVs for the present method range from

Table 3. Recovery of Added CRP and Interferences by Added Substances with CRP Binding to PE

Test	Test substance		CRP concn, mg/L: Initial	Added	Final	n	Recovery, %
a	Serum components		1 (1)	60	64 (5)	10	105
			30 (25)	60	89 (18)	19	98
b	Rheumatoid factor		25 (23)	60	81 (21)	10	93
c	Hyperlipemic serum		37	125	177	1	112
d	Human IgG, g/L	9.6	45 (33)	—	51.7 (46)	4	115
		96.0	45 (33)	—	55.3 (49)	4	123
e	Human hemoglobin, g/L	0.7	8 (10)	—	8.0 (10)	2	100
		1.4	8 (10)	—	8.8 (10)	2	111
		3.7	8 (10)	—	6.3 (9)	2	78
f	Human SAP, mg/L	4	22	—	24.9	1	113
		13	22	—	22.9	1	104
		22	22	—	22.5	1	102
g	Heparin, int. units/L	15 000	10	—	10.2	1	102
		20 000	10	—	11.2	1	112
	Citrate, g/L	1.6	10	—	9.8	1	98
		3.2	10	—	9.8	1	98
		6.4	10	—	9.0	1	98
	EDTA, g/L	1.0	10	—	9.1	1	91
		1.5	10	—	9.8	1	98
h	Serum dilutions	1/961–1/96	7 (12)	—	4.8 (10)	9	68
		1/961–1/480	15	—	10.8	1	72
		1/961–1/480	36	—	33.8	1	94
		1/961–1/480	92	—	80.9	1	88

Each sample was tested in duplicate, in a single experiment for each set of data (a to h). CRP concentrations are given as mean (SD) when different samples (n) were used. Other details as described in *Materials and Methods*.

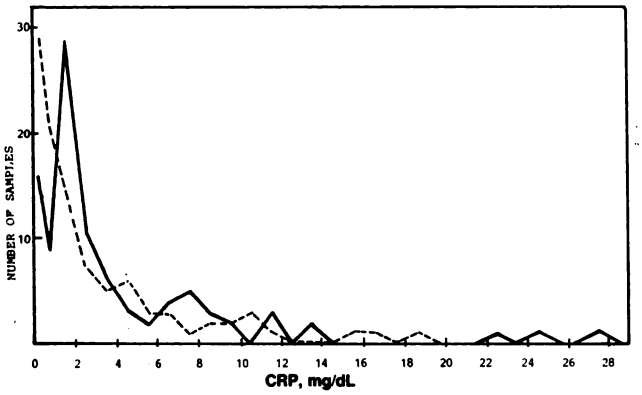

Fig. 4. Comparison of CRP concentrations in 99 sera as determined by nephelometry (----) and by CRP binding to PE (—)

The blood samples were allowed to clot and sera was separated and frozen until required. All these samples were classified as CRP-positive by latex test, performed as indicated in *Materials and Methods*. The samples were distributed in 10 mg/L classes, with 0–9 being the lowest grouping

Table 4. Correlations between CRP Binding to PE (*y*) and Other Methods for Quantifying CRP (*x*)

Comparisons	n	*r*	Regression equation
Lab 1			
c vs *d*	30	0.90	$y = 0.87x + 4.1$ mg/L
b vs *d*	66	0.89	$y = 1.14x + 2.2$ mg/L
c vs *d*	99	0.96	$y = 1.30x + 3.0$ mg/L
Lab 2			
c vs *d*	8	0.94	$y = 0.83x + 6.1$ mg/L
c vs *d*	41	0.90	$y = 1.08x + 12.7$ mg/L

A total of 244 independent measurements of 148 individual sera were made by: (*d*) CRP binding to PE, (*b*) pneumococcus-based ELISA, and (*c*) nephelometry. Lab. 1: the quality-control laboratory at Invesgen. Lab. 2: the microbiology laboratory at La Paz Hospital (both in Madrid). The different nephelometry assays were performed by different analysts.

4% to 7.5% (Table 1), as compared with 4.5% to 9.3% for method *c* (*18*). The between-run CV for method *d* ranges from 3.7% to 11.2% (Table 1 and Table 2), compared with 6.6% to 11.6% for method *c* (*18*).

The fact that there are lower between-run CVs in Table 2 than the between-run CVs on Table 1 could be the result of the larger n (8 in Table 1, 14–50 in Table 2). Between-run CVs in Table 2 are very similar to the within-run CVs in Table 1 (n = 54).

The present method offers a low incidence of false positives. When CRP-negative sera were assayed in within-run (n = 54) or between-run (n = 8 × 2) experiments, no false positives were obtained (Table 1). No data on this have been reported for methods *a*, *b*, or *c*.

All the characteristics mentioned above make the present assay method suitable for reliable quantification of normal and pathological CRP concentrations in serum, plasma, or other biofluids.

The cutoff value for distinguishing negative from positive by this technique, evaluated graphically, is 10 mg/L (Figure 2*A*). The 95% confidence limit, assuming normal distribution (Figure 2*B*), was 10.4 mg/L (n = 82). By radial immunodiffusion (*1*), CRP concentrations in normal healthy serum were ≤2 mg/L (374 healthy patients) but concentrations of 10 mg/L were also found. For EMIT (Syva Co.) assays (*17*) ≤18 mg/L is defined as normal (103 healthy patients).

Table 5. Qualitative Comparison between CRP Binding to PE (*d*) and Other Methods (*a, b, c*)[a]

	Comparison methods			
	a	*b*	*c*	*c'*
r	—	0.89	0.90	0.96
Sensitivity, %	79	100	91	95
Specificity, %	100	89	71	51
n	92	66	30	99

[a] The cutoff value between negatives and positives was 6 mg/L. Results of the comparison methods were taken as true, for purposes of quantifying sensitivity and specificity.

Sensitivity = [(true positives − false negatives)/true positives] × 100

Specificity = [(true negatives − false positives)/true negatives] × 100

By nephelometry (*18*) the normal concentration was ≤22 mg/L (61 healthy persons). In another, similar study this value was ≤6 mg/L (n = 60) (*11*). Using CRP quantification as a marker in sera of patients with colorectal carcinoma gave a cutoff value of 10 mg/L (*2*). By radioimmunoassay (*19*), CRP concentrations in all healthy adult sera were ≤8.2 mg/L (n = 153). Even if most of the normal samples contain CRP in the 2 to 4 mg/L range, the cutoff value should be higher than that, there being no clinical significance attached to low values in normal adults. Therefore the present method of quantification as well as the other methods referred above are sufficiently consistent that we can recommend 10 mg/L as the cutoff value for CRP concentrations in healthy subjects.

By using PE ligand in the solid phase and highly purified anti-CRP antibody in the conjugate, we have eliminated most of the possible and common interferences in the determination of CRP. Table 3 shows the absence of gross interferences from normal serum components, rheumatoid factor, hyperlipemic serum, IgG, hemoglobin, and SAP. Added heparin, citrate, or EDTA, to obtain plasma, did not interfere with the assay. Method *c*, on the other hand, is subject to interferences from hyperlipemic serum; no other possible interferences have been studied with regard to methods *b* or *c* (*11*).

In all, 148 different sera were analyzed by methods *a, b, c,* and *d* (Tables 4 and 5). The correlations, slopes, and intercepts between these assays and their respective means indicate that CRP binding to PE is a useful technique for determining CRP concentrations in clinical specimens.

The sera shown to be positive by methods *b* and *c* were respectively 91% and 100% positive by method *d*. However, only 79% of the sera positive by *a* were positive by *d*, most probably due to the high incidence of false positives in method *a* (*1*). The sera negative by *c* were also between 51 and 71% negative by *d*, reflecting the fact that all values by *c* were slightly lower than the values by *d*. This discrepancy might be due to differences in the calibrators or, more probably, to the low dilution–higher background in *c* (sera are diluted sixfold in *c*, 961-fold in *d*). Similar problems are found at lower dilutions in *d* (Table 3: results for 96-fold dilutions are 68% of those for 961-fold diluted samples).

With this new method we could assay 41 serum samples for CRP, in duplicate, in about 3 h (a rate similar to method *b*). In comparison with method *c* the assay time for 40 specimens in single determinations is about 2 h.

Ten-microliter samples of serum suffice for making the measurements, similar to that for method *b*, but better than the 1–2 mL required in method *c*. This micro-sample volume requirement, a result of the assay sensitivity, is especially desirable for testing sera from children. In addition such samples as broncho-alveolar lavage fluid, cerebrospinal fluid, and cell culture supernates may now be assayed with great precision (*1*).

Even though this study includes some preliminary data concerning the clinical relevance of the binding of CRP to PE-coated plates, a study involving a larger number of samples is needed.

We thank Angeles Samino Moreno for her technical assistance.

References

1. Morley JJ, Kushner I. Serum C-reactive protein levels in disease. In: Kushner I, Volanakis JE, Gewurz H, eds. C-reactive protein and the plasma protein response to tissue injury. Ann NY Acad Sci 1982;389:406–17.

2. Mercer DW, Talamo TS. Multiple markers of malignancy in sera of patients with colorectal carcinoma: preliminary clinical studies. Clin Chem 1985;31:1824–8.

3. Kushner I, Broder ML, Karp D. Control of the acute phase response. Serum C-reactive protein kinetics after acute myocardial infarction. J Clin Invest 1978;61:235–42.

4. Fisher CL, Gill C, Forrester MG, Nakamura R. Quantitation of acute phase proteins postoperatively. Value in detection and monitoring of complications. Am J Clin Pathol 1976;66:840–6.

5. Peltola HO. C-reactive protein for rapid monitoring of infections of the central nervous system. Lancet 1982;i:980–2.

6. Mortensen RF, Rudczynski AB. Prognostic significance serum CRP levels and lymphoid cell infiltrates in human breast cancer. Oncology 1982;39:129–33.

7. Lariviere F, Dolce P, Masson M, Nadeau L. C-reactive protein as biochemical indicator of bacterial infection in neonates. Clin Biochem 1986;9:192–8.

8. Pepys MB. C-reactive protein fifty years on. Lancet 1981;ii:653–7.

9. Pontet MA, Ayrault-Jarrier M, Burdin J, Gelin M, Engler R. Préparation et forme sérique de la protéine réactive C de lapin. Biochimie 1979;61:1293–9.

10. Young NM, Williams RE. Comparison of the secondary structures and binding sites of C-reactive protein and the phosphorylcholine binding murine myeloma proteins. J Immunol 1978;121:1893–8.

11. C-reactive protein (CRP). Immunochemistry systems (ICS). Fullerton, CA: Beckman Instruments Inc., 1986.

12. Tijssen P. Practice and theory of enzyme immunoassays. In: Burdon RH, Van Knippenberg PH, eds. Laboratory techniques in biochemistry and molecular biology. Amsterdam: Elsevier, 1985:15.

13. Calvin JK, Burling C, Blow I, Barnes P, Price CP. Evaluation of fluorescence excitation transfer immunoassay for measurement of specific proteins. J Immunol Methods 1986;86:249–56.

14. Blanck DW, Kroll MH, Rudell ME, Elin RJ. Hemoglobin interference from in vivo hemolysis. Clin Chem 1985;31:1566–9.

15. Pepys MB, Baltz ML, De Beer FC, et al. Biology of serum amyloid component. In: Kushner I, Volanakis JE, Gewurz H, eds. C-reactive protein and the plasma protein response to tissue injury. Ann NY Acad Sci 1982;389:286–98.

16. Dacie JV. Lewis SM. Practical haematology, 6th ed. Edinburgh: Churchill Livingstone, 1984:43–7.

17. C-reactive protein assay. Syva Advance™ automated fluorescence immunoassay system. Palo Alto, CA: Syva Co., 1983.

18. Las R. Human CRP test kit for quantification of human C-reactive protein (CRP) in serum. Hyland Diagnostic, 1983.

19. Claus DR, Osmand AP, Gewurz H. Radioimmunoassay of human C-reactive protein and levels in normal sera. J Lab Clin Med 1976;8:120–8.

20. Rodbard D. Statistical estimation of the minimal detectable concentration (sensitivity) for radioligand assays. Anal Biochem 1978;90:1–2.