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Development of a Fast Solid-Phase Enzyme Immunoassay for C-Reactive Protein

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A fast sandwich enzyme immunoassay has been developed for C-reactive protein (CRP). This method can be used for screening CRP concentration in large numbers of samples providing a non precipitation, non agglutination and non radioactive alternative for assessment of human CRP. Advantages over previously reported CRP sandwich assays include: assay time was reduced from 4 1/2 h to 45 min, incubations were made at room temperature instead of 37 °C and serum dilutions required were 100-400 fold instead of 10000 - 20000 fold. Correlations were good with both nephelometry and phosphorylethanolamine binding assay. The 45 % false positives found with the slide-latex anti C-reactive protein method were reduced to 0 % by the use of the described method.

Key words: C-reactive protein, Solid-phase ELISA Fast.

C-reactive protein (CRP) is a blood serum protein whose concentration increases from 0.5-1 mg/dl in healthy individuals, up to 50 mg/dl in different pathological states (11, 13). The CRP test is mainly used in neonatal infections (8), tumor evolutions (10), post-operative evolutions (11), and rheumatoid arthritis (13).

Quantitation of CRP is generally carried out after fast screening of large number of samples by commercially available slide-latex anti-CRP tests. But the latex test is characterized both by a high number of false positives and by a loss of positives with high CRP content because of the prozone phenomena (3). Recent methods for quantitation of CRP include radioimmunoassay (2), nephelometry, phosphorylcholine binding assay (15), automated particle counting assay (5), and solid-phase enzyme immunoassay (7).

Solid-phase enzyme immunoassays are increasingly being used for detecting proteins in a variety of applications (16). A general approach for the detection of proteins is to create a so-called polyclonal antibody sandwich (immobilized antibody, antigen, antibody conjugated to an enzyme). The Solid-phase enzyme immunoassay described to quantify CRP is a sandwich immunoassay (7) with a high sensitivity and free from rheumatoid factor interferences but it requires 4 1/2 hours to complete, extreme dilutions of

serum (1/10000-1/20000) and incubations at 37 °C. By optimization of Solid-phase coating, antibody conjugation and length of the assay, simplifications have been worked out to avoid the long incubation time, high dilution of samples and incubations at 37 °C.

Materials and Methods

CRP purification. — The method of PONTET et al. (14), was used to purify CRP. Briefly, CRP was purified from human ascitic fluid by affinity chromatography onto agarose immobilized 2-aminoethyl-dihydrogen phosphate (Pierce, Ill, USA). CRP purification was monitored by immunodiffusion, which was carried out in 200 mM borate, 75 mM NaCl, 2.5 mM $CaCl_2$, 1 % agar, 100 mg/l merthiolate, pH 8. CRP concentrations were calculated from A280 nm and ε 1 % 19.5. Polyacrylamide gel electrophoresis showed one band at 21 KDa in the presence of 1 % SDS and β-mercaptoethanol (9).

Preparation of conjugates. — The conjugates used for most experiments were obtained by one step glutaraldehyde method as described by GUEDSON and AVRAMEAS (6). Anti-CRP goat serum was obtained from Difco (USA), and Biomerieux (France). Anti-CRP rabbit serum was obtained from Llorente (Spain). Specific anti-CRP was obtained by passing the serum through a CRP-Sepharose column. About 10 ml of antiserum were passed through an 1.5 × 5 cm Sepharose column (5 mg of CRP/ml of agarose). Bound anti-CRP was eluted with 50 mM ethylenediamine pH 11, neutralized immediately by collecting the 1000 µl fractions on 100 µl of Tris 3 M pH 5. The yield was about 1-2 mg of specific anti-CRP per ml of antiserum. Two single bands at 50 KDa and 25 KDa were resolved on polyacrylamide gel electrophoresis. To obtain IgG with anti-CRP activity, antiserum was diluted 2 fold with 0.01 M sodium phosphate, 0.15 sodium chloride (PBS) pH 7.2 and made 50 % ammonium sulfate by adding an equal volume of saturated solution. The pellet after 1 h at 37 °C and centrifugation, was washed with 45 % and 40 % of ammonium sulfate in PBS. The final pellet was dialysed against 15 mM sodium phosphate buffer pH 7.8. This fraction was passed through a DEAE cellulose column (9.6 × 2.5 cm Cellex D, Bio-Rad) and the eluted fractions were passed through a human pooled sera affinity column 6 × 2.5 (60 mg of human serum negative for CRP plus 1 g of activated CNBr from Sepharose, Pharmacia). The amount of IgG was estimated by using $\varepsilon \frac{1\%}{280} = 14$. The human pooled serum was made negative for CRP by passing it through the agarose immobilized 2-aminoethyldi-hydrogen phosphate column (Pierce).

To couple peroxidase by the method of the one-step glutaraldehyde (4), 8.4 mg of lyophilized anti-CRP were mixed with 5-10 mg of horseradish peroxidase (Seravac Miles, RZ = 3.0) in 0.7 ml of PBS pH 7.4 and 0.25 % glutaraldehyde. After incubation at room temperature for 3 h, 40 µl of 2 M glycine were added and incubated overnight at room temperature. The final reaction mixture was passed through a Sephadex G-200 1 × 30 cm column in 0.5 M PBS pH 7.4. Fractions eluting at the void volume were pooled together and kept at 4 °C. The resulting conjugates were about 50-fold concentrated from the dilution of use (absorbance of 1.5 of 492 nm at 160 ng/well CRP). The molar ratio of antibody to peroxidase was 1 to 0.91 calculated by $\varepsilon \frac{2\ \%}{280} = 1.4$ for antibody and $\varepsilon \frac{1\ \text{mM}}{403} = 80$ for peroxidase. To couple peroxidase to IgG by the method of NAKANE and KAWAOI (12), 5 mg of peroxi-

dase in 1 ml of 300 mM $NaHCO_3$ pH 8.1, were mixed with 0.1 ml of 1 % dinitrofluorobenzene in ethanol for 1 h at room temperature; then 1 ml of 80 mM $NaIO_4$ were added and mixed for 1 h. The activated peroxidase was dialyzed against 10 mM sodium carbonate-buffer pH 9.5. Five mg of IgG in 1 ml of 10 mM carbonate-buffer were mixed with the activated peroxidase for 3 h and then 5 mg $NaBH_4$ was added and further incubated overnight at 4 °C. After dialysis against PBS, the resulting conjugates were 10-fold concentrated from the dilution of use. Conjugates were kept in PBS at 4 °C until use.

Correlation studies. — The blood samples were allowed to clot and sera was separated by centrifugation and frozen until use.

Samples were assayed by rate nephelometric immunoassay using a Beckman ICS with ICS reagents according to the manufacturer's instructions. Samples were assayed by binding to phosphorylethanolamine as described previously (3).

CRP sandwich enzyme immunoassay. — Polystyrene wells (Costar) were coated with 1-2 µg/well of purified IgG, diluted in distilled water, from polyclonal anti-CRP or by the corresponding amount of antisera anti-CRP (assuming a concentration of 15 mg of IgG/ml of antisera). One hundred µl of anti-CRP diluted in distilled water were either dried overnight or incubated in a humid chamber for 3-5 h at 37 °C, respectively. After this step, the wells were washed in 30-fold diluted dilution buffer, dried at 37 °C and kept sealed under vacuum at 4 °C.

The CRP sandwich enzyme immunoassay was made as follows: 50 µl of sample 100-400 fold diluted in dilution buffer (0.2 M borate, 75 mM NaCl, 2 mM $CaCl_2$, 1 % Bovine serum albumin, 0.01 % merthiolate, 0.05 % Tween 20, pH 8) were added per well and incubated at room temperature for 15 minutes. After washing with distilled water, 50 µl of conjugated anti-CRP diluted 1000 fold in dilution buffer were added per well and incubated for 15 minutes. The plates were then washed 4 times with distilled water (1) 50 µl of substrate buffer added and reaction stopped with 50 µl of H_2SO_4 6 N after 15 minutes. The contents of the wells were read in a Titertek Multiskan at 492 nm adjusting the zero to the air. Under thes conditions 100 µl of water gave an absorbance of 0.03. All the steps were carried out at room temperature. Maximal signals were measured at CRP concentrations of 160 ng/well near the level of saturation of the assay. Backgrounds were measured with dilution buffer alone.

The single step procedure was performed as follows: 25 µl of diluted samples were added to wells, and then 25 µl of anti-CRP peroxidase were added and mixed. Incubation was allowed to proceed for 15 minutes and after washing 4 times, substrate buffer was added. All the steps were carried out at room temperature.

Results

Coating with anti-CRP. — Methods of coating by evaporation to dryness or incubation in the humid at 37 °C together with the use of whole antisera or affinity purified anti-CRP were compared (figure 1). After coating, some wells were treated with glutaraldehyde. By evaporation to dryness, only affinity purified anti-CRP gave a signal proportional to the amount of CRP (figure 1A and A') whereas by incubation in the humid both whole antisera and purified anti-CRP did (figure 1B and B'). In both the above mentioned cases, the backgrounds were lower by using affinity purified anti-CRP. Treatment with glutaraldehyde resulted in loss of activity in the wells coated by incubation in the humid whereas the same treatment preserved 50 percent of the activity in the

wells coated by evaporation to dryness (not shown).

The minimum time of incubation of whole antisera to obtain a stable and reproducible coating is about 2 hours. After this time there were no changes in coating efficiency. Backgrounds were 6 % by using distilled water and 8 % by using sodium carbonate buffer (not shown).

Coated plates were either stable when kept dried or lost 70 % of their activity when kept in humid chambers during a 7 day exposure to 37 °C. Plates coated with either whole antisera (by humid incubation) or purified IgG (by evaporation to dryness), conserved 100 % of the activity when kept under vacuum at 4 °C during 6-8 months (not shown).

Table I shows that variation coefficient could be lowered from 24.5 % to about 8-10 % by blocking with dilution buffer at several dilutions. Background percentage which was 19.8 % without any blocking, could also be lowered to 8 - 9 % by the

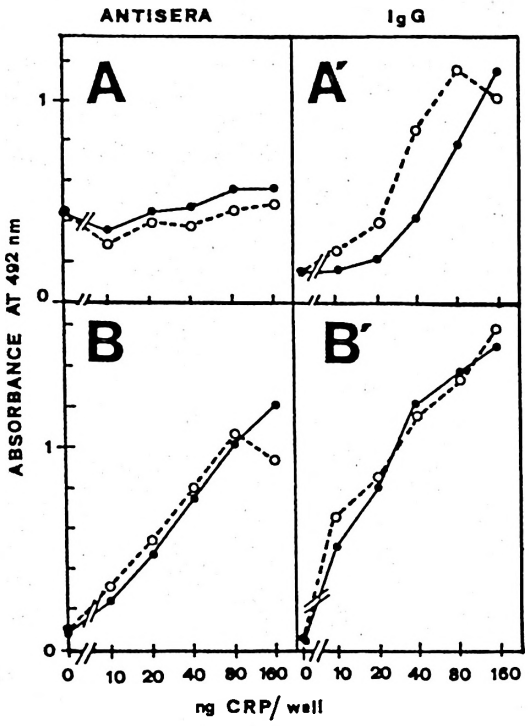

Fig. 1. *Coating with antisera (A, B) or IgG (A', B') anti CRP.*
Costar plates were coated with amounts equivalent to 2.5 (o) or 5 (•) µg of IgG/100 µl of water/well by different methods. Whole diluted goat antiserum anti-CRP was used in A and B. Specific IgG anti-CRP was used in A' and B'. After adding anti-CRP to the wells, they were either incubated at 37 °C 16 h to dryness and washed with 1/20 dilution buffer prior to assay (experiments A, A') or incubated at 37 °C 5 hours in a humid chamber and then washed with 1/20 dilution buffer prior to assay (experiments B, B').

Table I. *Influence of post-coating treatment in the intraassay variation and percentage of background.* Wells were coated by diluted anti-CRP goat anti-serum (equivalent to about 2 µg IgG/well). After coating, blocking was made by 15 minutes incubation with the above referred buffers, then washed with 50-fold diluted dilution buffer and dried. Conjugate used was obtained with whole antiserum. Variation coefficient (CV) was, S.D./average × 100 and percentage of background was, average absorbance without CRP/average absorbance with CRP × 100. Number of wells used = 48.

Post-coating treatment	Intraassay, CV		Background %
	-CRP	+CRP	
No treatment	25.7	24.5	19.8
50-fold diluted dilution buffer + 1 % gelatine	36.7	9.8	16.8
50-fold diluted dilution buffer	19.2	10.0	9.6
2-fold diluted dilution buffer	21.8	8.2	9.0
Dilution buffer	19.0	8.3	8.6

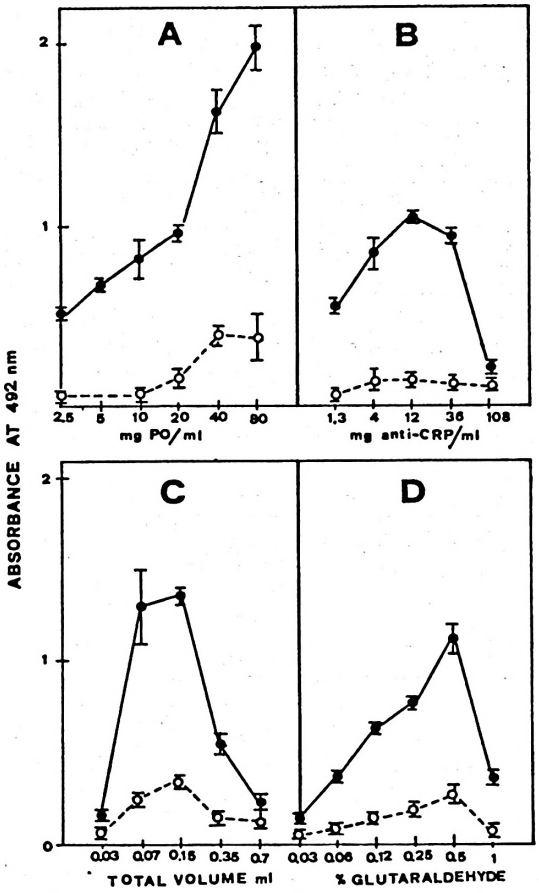

Fig. 2. *Optimization of conjugation of peroxidase to whole anti-CRP antisera.*
Peroxidase concentration (A), antisera concentration (B), total dilution (C) and glutaraldehyde concentration (D). Conjugation was with 0.25 % glutaraldehyde during 3 h at room temperature. Total volume of reaction was 100 µl of 10 mM sodium phosphate 150 mM NaCl, pH 7.4. After conjugation 4 µl of 2 M glycine were added and left overnight. Conjugates were diluted 1:1 with water and assayed at 1/400 dilution in the presence (•) or in the absence (o) of CRP. Points are averaged from duplicate. In experiment A, antisera concentration was 12 mg of protein/mal. In experiment B, peroxidase concentration was 10 mg of protein/ml. In experiment C, antisera was 1.2 mg and peroxidase 1 mg in different volumes and then assayed at the same final dilution. In experiment D, antisera concentration was 12 mg of protein/ml and peroxidase concentration was 10 mg of protein/ml.

same treatment as above. The inclusion of gelatine in the blocking buffer did not show any improvement. Similar results were obtained by using commercial antisera from Difco or Biomerieux.

Anti-CRP conjugation to horseradish peroxidase. — Immunoconjugates with peroxidase of the highest purity and specific activity (RZ = 3) were prepared by the use of glutaraldehyde or periodate.

The one step glutaraldehyde method was optimized by using whole antisera. All these variables were optimized: peroxidase concentration, antisera concentration, total volume and glutaraldehyde concentration (fig. 2). The total activity of the conjugate increased as peroxidase was increased, however background also increased from about 6 % to 20 % at 10 and 80 mg of peroxidase per ml, respectively. The activity of the conjugate increased with the antisera concentration to a maximum at about 12 mg of protein/ml. Total volume of the reaction mixture was equivalent to total concentrations of protein (peroxidase and antisera), of 66, 28, 13, 5.6 and 2.8 mg/ml. At 66 mg/ml the conjugate was solid so that only supernatant was assayed. The activity increased to a maximum at 28-13 mg of protein/ml.

Optimal concentrations for coupling were, therefore, 10 mg of peroxidase/ml, 12 mg of protein antisera/ml and 0.25-0.5 % glutaraldehyde (fig. 2). The results obtained in small batches were confirmed by using reactions up to 1 ml of total volume. Horseradish peroxidase from Sigma (265 U/mg, RZ 3.1) and Miles (248 U/mg, RZ = 3.2) gave similar results.

The conjugates obtained by using whole antisera as source of anti-CRP have backgrounds of 20-25 % (fig. 2). To lower the background of the conjugates, purification of antisera prior to conjugation was performed by affinity chromatography on CRP columns (specific anti-CRP) and by ammonium sulfate precipitation and DEAE chromatography (purified IgG

Table II. *Percentage of background of anti-CRP antibodies conjugated to peroxidase.* Methods used to obtain the conjugate were G-glutaraldehyde and P-periodate. After the coupling reaction, Sephadex G-200 chromatography separated high molecular weight molecules from free peroxidase. The assay method was performed in plates coated with whole antiserum, purified IgG or phosphorylethanolamine (PE). Ranges of percentage of background from 3-4 different experiments are given. Percentage of background was calculated by absorbance in the absence of CRP/absorbance in the presence of CRP × 100.

Anti-CRP antibodies		Method of conjugation	Purification of conjugate	Assay method (coating)	Percentage of background
Source	Purification				
Goat	—	G	—	Antiserum	20-25
Goat	—	G	—	IgG	10-12
Goat	Affinity chromatography	G	—	PE	10-13
Goat	Affinity chromatography	G	G-200	PE	5-9
Rabbit	Amm. sulphate + DEAE	P	—	PE	8-15
Rabbit	Amm. sulphate + DEAE	P	G-200	PE	1-4
Rabbit	Amm. sulphate + DEAE	P	G-200	IgG	3-5

with about 10 % of specific anti-CRP as demonstrated by affinity chromatography purification). Since the background could also be due to unspecific binding of human proteins, the antisera to be used as a source of specific anti-CRP was passed through an affinity column made by covalent attachment of CRP-free human serum to Sepharose. About 25 % of the total IgG was eluted from such a column (not shown). By using purified fractions in coupling and maintaining whole antisera for coating the optimal concentration during coupling of purified IgG to obtain a conjugate with maximal titre was about 5 mg/ml with 11 % of background (not shown).

To discriminate the background produced by conjugate from the one produced by coating, the purified fractions after conjugation were assayed by binding to phosphorylethanolamine. Purification of the conjugate from affinity purified anti-CRP, after the coupling reaction with glutaraldehyde by chromatography on G-200 Sephadex reduced the background from 10-13 % to 5.9 %. Purification of the conjugate from total IgG anti-CRP after the coupling with periodate by chromatography on G-200 Sephadex reduced the background from 8-15 % to 1.4 %. Similar results were observed by using solid-phase coated with IgG anti-CRP (table II).

Titre of conjugate was defined as the dilution at which an absorbance of 1.5 at 492 nm was obtained in the sandwich CRP assay at 16 ng CRP/well. The yield of conjugate was calculated and expressed as ml of conjugate of a titre of 1/1000 per ml of antiserum. Using goat antiserum anti-CRP coupled by the one step glutaraldehyde method a yield of 2.5 was obtained. Similar yields were obtained by using either the glutaraldehyde method for specific anti-CRP purified by affinity chromatography or the periodate method for total purified IgG (2 and 1.5 ml of conjugate 1/1000/ml of original antiserum). No active conjugate was obtained by us-

ing the glutaraldehyde method with purified Fab'2 obtained from total purified anti-CRP IgG (not shown). Linearity could be extended up to CRP concentrations of 750 ng/well by using 125-fold diluted conjugate. Linearity was good between 10-150 ng CRP/well and percentage of background was minimal at 500-fold dilution of conjugate (not shown).

The conjugates obtained by either the glutaraldehyde or the periodate method were stable for 8-12 months when kept 10-fold concentrated in dilution buffer at 4 °C no matter what the purity of the anti-CRP was used for conjugation (data not shown).

Shortening the length of the assay. — The maximal binding of CRP to Solid-phase anti-CRP is achieved at 15 minutes of incubation (figure 3). No differences were found in the time course of the assay when performed at room temperature (20-24 °C) or at 37 °C. By increasing the time of incubation with conjugate, the absorbance was proportionally increased up to 60 min (not shown). Figure 4 shows the time course of the peroxidase-*o*-phenylene-diamine reaction. With free peroxidase or free antibody-peroxidase conjugate the absorbance kept increasing up to 60 min. The absorbance with the antibody-peroxidase bound to the complex Solid-phase-antibody-CRP increased linearly only for the first 30 minutes.

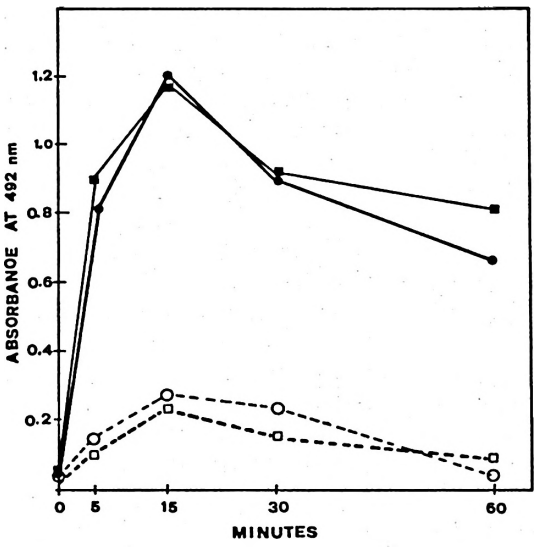

Fig. 3. *Time course of CRP binding to Solid-phase anti-CRP in the presence (●, ■) or in the absence (○, □) of CRP.*
Wells coated with 1 µg of affinity purified anti-CRP were incubated at room temperature (●, ○) or at 37 °C (■, □) at the times indicated in the figure. CRP concentration (●, ■) was 200 ng/well. Other details of the assay were as indicated in Materials and Methods. Averages from duplicates are given.

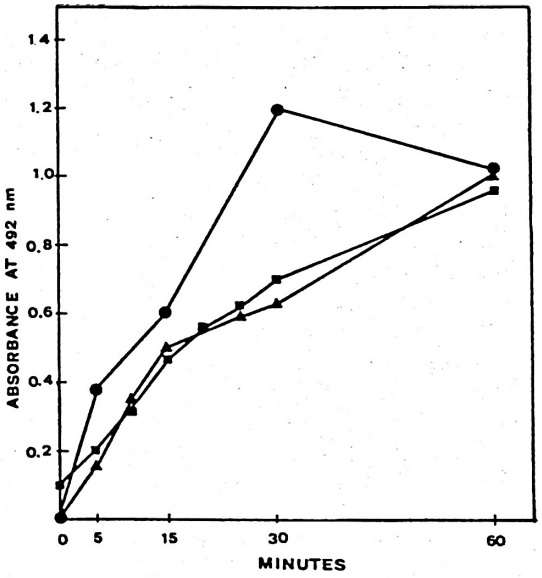

Fig. 4. *Time course of free peroxidase (■), conjugated peroxidase (▲) and colour development in ELISA (●).*
Ten microliters of 50 ng/ml of peroxidase were incubated with 50 µl of substrate buffer for the time periods indicated in the figure and reaction stopped with 4 N H_2SO_4 (absorbance values have been multiplied by a factor of 2 to make the results comparable with the rest). Ten microliters of peroxidase conjugated to anti-CRP diluted 4000-fold were assayed and reaction stopped as above. CRP-ELISA was performed as indicated in Materials and Methods. Averages from duplicates are given.

Table III. *Comparison of serum samples classified by the sandwich CRP ELISA and other methods.* (Cut off between positives and negatives was considered at 0.6 mg/dl of CRP.) The samples were classified as double positives or negatives when the results coincided in both methods, as positive-negative when the results were positive by the sandwich CRP method and negative by the other method and as negative-positive when the results were negative by the sandwich CRP method and positive by the other method. The number of samples used for comparison were 20. Nephelometry measurements were made with a Beckman ICS spectrophotometer. CRP binding to phosphorylethanolamine (PE) was made as described (3,4).

Methods of comparison	Percentage of the total number of samples			
	Double positives	Double negatives	Positive-negatives (sensitivity)	Negative-positives (specificity)
Nephelometry	55	45	—	—
Binding to PE	55	45	—	—
Slide-latex	55	—	—	45

Sandwich CRP-ELISA made by simultaneous rather than stepwise incubation of sample and conjugate showed similar results. However the simultaneous assay was linear only up to 160 ng CRP/well. When more than 160 ng CRP/well were used, the activity decreased and higher concentrations completely abolished all activity (not shown).

A time reduction could also be obtained by reducing the number of washings. Initially 2 and 4 washings were used following sample and conjugate incubations, respectively. By reducing the number of washings to 1 and 3, respectively, no higher backgrounds were detected, provided that plates were strongly shaken to eliminate small drops. A reduction of about 40 % in the absorbance was obtained when distilled water was used instead of 50-fold diluted dilution buffer but backgrounds also dropped. The elimination of Tween in the washing buffer increased both signal and background proportionally (not shown).

Comparison with other methods. — By using optimized conditions, the number of samples classified as negative or positive by the sandwich CRP-ELISA by visual criteria were identical with the data obtained by nephelometry and CRP binding to PE (cut-off between positives and negatives at 0.6 mg/dl). Discrepancies were only obtained with the slide-latex test due to its high incidence of false positives (table III).

Discussion

Althougth the use of reagents of the highest purity is preferred for quantitation by enzyme immunoassays (6, 12, 16), whole unpurified hyperimmune anti-CRP antisera could be used to develop a sandwhich enzyme immunoassay to qualitatively measure human CRP.

Coating of Solid-phase should be performed by incubating anti-CRP antisera dilutions with the Solid-phase in humid chambers (4). Most probably, interfering proteins do not allow the coating by dryness, since this method only worked when purified anti-CRP IgG was used (fig. 1). Conditions to obtain peroxidase conjugates by using whole antisera were also optimized (fig. 2). By using whole anti-CRP antisera both for coating the microtiter wells and for obtaining the conjugate, any laboratory could adapt the assay with little or no equipment. Best results are obtained, however, with purified IgG from anti-CRP antisera for both coating and conjugate (table II and fig. 1).

Background lowering was obtained by,

purification of conjugates both before and after the coupling (table II), optimization of the coupling reaction (fig. 2), selection of the coating buffer purification of anti-CRP for coating and using blocking buffer (table I). Backgrounds decreased to 0.06-0.08 A 492 nm, corresponding to a 4-5 %, which were lower than in the reported phosphorylcholine binding assay (15), the automated particle counting assay (5), the solid-phase enzyme immunoassay (7) or the rephelometry (not shown).

Optimal total time of the enzyme immunoassays depends on duration and number of both incubations and washings. Since no optimization of timing is generally studied, enzyme immunoassays are very often longer than needed, total time varying from 2 to 4 h (16). Recently reported solid-phase enzyme immunoassays for CRP suffer from this disadvantage together with the need for inconvenient 10000-20000-fold serum dilutions and 37 °C incubations (7) while we carried out a similar assay for CRP with 45 minute total incubation time, and at room temperature. Time for diluting the samples has also been reduced on account of the ease in obtaining lower-fold dilutions (100-400-fold).

A method for screening large numbers of samples must have certain fundamental characteristics for clinical utilization, such as: simplicity, sensitivity, and possible automation. The method described here offers some of these advantages together with high specifity.

Resumen

Se desarrolla un enzimoinmunoensayo *sandwich*, para la proteína C-reactiva de ejecución rápida, que puede usarse para una primera valoración de la proteína-c-reactiva (PCR) en gran número de muestras, aportando una alternativa ni precipitante, ni aglutinante, ni radiactiva, para la estimación de la PCR humana. Sus ventajas sobre otros enzimoinmunoensayos *sandwich* de CRP incluyen: la reducción del tiempo del ensayo (de 4 1/2 h a 45 min), incubaciones a temperatura ambiente en lugar de 37 °C, y diluciones de las muestras 100-400 veces en vez de 10.000-20.000 veces. Las correlaciones con la nefelometría y con el ensayo de ligamiento a la fosforiletanolamina son buenas. El 45 % de falsos positivos encontrados con el uso del método látex se pueden reducir al 0 % mediante el uso del método descrito.

Palabras clave: Proteína c-reactiva, ELISA en fase sólida, Rápido.

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