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Heme increases peroxidase-antibody activity in aged conjugates

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Decay of activity in antibody-peroxidase conjugates is faster when highly diluted as in clinical kits. Following aging at 4° C, some of this loss of activity can be restored by incubation with heme just before use. The addition of heme in the conjugate storage buffer prevents some decay of activity and is not due to the pseudoperoxidase activity of heme. This procedure permits conjugates to be presented in an easy ready-to-use form for clinical immunoenzymatic assays.

Key words: Stability; Peroxidase conjugate; Heme

Introduction

For practical reasons, the clinical use of ELISA for antigen or antibody determination requires highly stable conjugates of antibody-enzyme that can be stored at 4° C and at ready-to-use highly diluted concentrations (Martinez and Coll, 1987). Diluted peroxidase conjugates have a more rapid decay of conjugate activity than 100–1000-fold concentrated preparations. Peroxidase (hydrogen-peroxide oxido-reductase, EC 1.11.1.7.) is a hemin glycoprotein with a molecular weight of 40 000. It occurs as multiple isoenzymes with 1 mol of proto-hemin IX and 1 mol of iron as the prosthetic group. The covalent structure comprises two compact domains between which the hemin group is sandwiched and the concentration-dependent stability of peroxidase conjugates may be explained by the loss of hemin (J.F. Delagneau, personal communication). The concentration dependence of the loss of activity of peroxidase conjugates due to this mechanism has been investigated.

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Materials and methods

Heme isolation

Heme was isolated from frozen human red blood cells collected from clotted blood, by modifications of the method described by Connelly et al. (1958).

After thawing the clots, HCl was added to a final concentration of 0.4 N and stirred for 30 min. An equal volume of either chloroform or cyclohexanone was added to the mixture and agitation continued over 30 min. The organic phase was centrifuged or decanted and concentrated in a rotary evaporator.

Purity was checked by paper chromatography using toluene/acetic acid (12 : 1, v/v). The heme band exhibited pseudoperoxidase activity (brown colored) when the plates were sprayed with dimetoxybenzidine in methanol. The heme concentration was estimated by $\varepsilon^{1mM}_{403nm} = 80$ in ethanol (Dawson et al., 1969).

Dried heme was incorporated into the solution by sonicating in dilution buffer for 10 to 20 min (Virsonic 300 Sonicator Virtis). Alternatively, cyclohexanone concentrated heme was dissolved in ethanol (1 to 9 ratio), added to the dilution buffer and agitated until there was a complete solution (1 to 500 ratio).

Preparation of conjugates

The conjugates used for most experiments were obtained by a one-step glutaraldehyde method (Guesdon and Avrameas, 1983). Anti-human C-reactive protein (anti-CRP) goat serum was obtained from Bio-Mérieux (France). Specific anti-CRP was obtained by passing the serum through a CRP-Sepharose column. About 10 ml of antiserum were passed through a 1.5 × 5 cm Sepharose column containing 5 mg of CRP/ml of agarose. CRP was obtained as described previously (Martinez and Coll, 1987). Bound anti-CRP was eluted with 50 mM etylenediamine pH 11 and neutralized immediately by collecting 1 ml fractions into 100 μl of Tris 3 M pH 5. The yield was about 1 mg of specific anti-CRP/ml of antiserum. The preparation yielded two single bands at 50 kDa and 25 kDa when analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions.

Lyophilized anti-CRP IgG (2.4 mg) were mixed with 10 mg of horseradish peroxidase (Seravac Miles-Martín, Spain) in 0.7 ml of 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 and 2% glutaraldehyde. After incubation at 37 °C for 3 h, 40 μl of 2 M glycine were added and the mixture incubated overnight at room temperature. The final reaction mixture was passed through a column of Sephadex G-200 (1 × 30 cm) equilibrated in 0.01 M phosphate 0.5 M NaCl, pH 7.4 (phosphate-buffered saline, PBS). Fractions eluting in the void volume were pooled together and kept at 4 °C. The immunoglobulin concentration of the resulting conjugates was 340 μg/ml, and they were used after a further dilution of 1/50 (absorbance at 492 nm of 1.5 using 160 ng of CRP/ml in the CRP assay to be described later). The molar ratio of antibody to peroxidase was 0.91 to 1 calculated by $\varepsilon^{1\%}_{280nm} = 1.4$ for antibody and $\varepsilon^{1mM}_{403nm} = 80$ for peroxidase. The immunoglobulin content of the conjugates at the final dilution used was 6.8 μg/ml.

Incubation with heme and activity measurements

The conjugates were stored for different intervals (see figure legends) at several dilutions ready to use. Samples containing 34–68 μg/ml of immunoglobulin were diluted in a buffer having the following composition: 0.2 M borate, 2 mM $CaCl_2$, 1% bovine serum albumin (BSA), 100 mg/l merthiolate, 75 mM NaCl, 0.05% Tween 20, pH 8.3 whereas concentrated preparations containing 340–3400 μg/ml of immunoglobin were stored routinely in 1% BSA, 50% phosphate-buffered saline (PBS) in glycerol at pH 7.4 at −20 °C. Aged conjugates were incubated for 1 year at 37 °C with or without heme prepared as described before, prior to assay. The heme concentration and the incubation periods used in different experiments are described in figure legends.

To measure peroxidase activity, conjugates were further diluted (1/5 or 1/500 as appropriate) with dilution buffer after incubation with heme. 10 μl of diluted conjugates were incubated with 50 μl of 1 mg/ml *o*-phenylenediamine in 150 mM sodium citrate substrate buffer, pH 4.8 containing 3 mM H_2O_2 and stopped with 50 μl of 4 N SO_4H_2 at 4 min. Absorbance at 450 nm was measured with a Titertek Multiskan (Flow). Single point measurements were made.

To measure horse-radish peroxidase (HRP)-labelled anti-CRP activity, wells were coated with 0.25 μg CRP (Iturralde and Coll, 1984) and incubated with 50 μl of diluted conjugates for 30 min at room temperature. After washing four times with 1/20 diluted dilution buffer, bound HRP was measured as above. HRP-labelled anti-CRP activity was also measured in a commercial assay (INVESGEN, Madrid, Spain) in which CRP was immobilized on wells coated with phosphorylethanolamine. In this, wells were incubated with 100 μl of anti-CRP peroxidase for 30 min at room temperature. After washing five times, 50 μl of citrate buffer pH 4.8 with 3 mM H_2O_2 and 1 mg/ml of *o*-phenylenediamine were added and color development stopped with 4 N H_2SO_4 after 30 min. The wells were measured in a SLT EAR 400 FW spectrophotometer at 492–620 nm. Measurements were made by duplicate (Martinez and Coll, 1987).

Storage with heme and activity measurements

Diluted conjugate (68 μg/ml Ig concentration) was stored for several months (Table I, legend) in dilution buffer with or without 0.5 μM heme at 4 °C, and compared with less diluted conjugate (3.4 mg/ml Ig concentration) stored in PBS-glycerol buffer at −20 °C. Stored conjugates were

assayed by the CRP-binding assay as described above.

Results

Anti-CRP conjugated to peroxidase as described in the materials and methods section, lost 74% of its activity when stored diluted at 4° C in PBS pH 7.4 at concentrations of 34–340 μg/ml. No loss of activity, however, was measurable when the same conjugates were stored at −20° C in 1% BSA, 50% PBS pH 7.4, 50% glycerol at 3.4 mg/ml immunoglobulin concentration. To test the possible influence of heme concentration in these losses, two kinds of experiments were performed: (a) stored conjugates were incubated with heme for short periods and assayed, and (b) conjugates were stored for prolonged periods with or without the addition of heme and later assayed.

Fig. 1*A* shows the peroxidase activity of labelled anti-CRP after being kept at 34 μg/ml for 3 months in dilution buffer and incubated overnight in the absence or presence of increasing amounts of added heme. An increasing peroxidase activity levelling out at 2 μM final heme concentration was found in the four dilutions of conjugates tested. The maximum relative increment of activity of the conjugates incubated with heme over the conjugates incubated without heme was about: 2.2-fold for 6.8 μg/ml Igs (no dilution), 2.2-fold for 3.4 μg/ml Igs (1/2 dilution), 2.6-fold for 1.7 μg/ml Igs (1/4 dilution) and 2.6-fold for 0.85 μg/ml Igs (1/8 dilution). To investigate whether or not the increase was due to the added heme or to the peroxidase-antibody conjugates, the same experiment was analyzed in wells coated with CRP. Fig. 1*B* shows that similar increases were obtained when using this method of analysis. Furthermore heme alone did not have any measurable activity, up to 20 μM concentration (data not shown). The experiments reproduced in Figs. 1*A* and 1*B* were carried out simultaneously by single point measurements in the presence of 1 mM $FeCl_2$ to evaluate the influence of reduced iron in the reaction. No significant differences were detected.

In the absence of overnight incubation with heme, no increase of activity could be detected. Experiments made by adding cyclohexanone/ethanol dissolved heme instead of sonicated heme showed similar results on conjugates aged at 4° C at 340 μg/ml Ig concentration. Aged conjugates were incubated in the presence of 1.6 μM heme for 1 or 7 days to investigate the possible saturation of the reaction. Fig. 2 shows the activity of conjugates in the CRP-binding assay with differ-

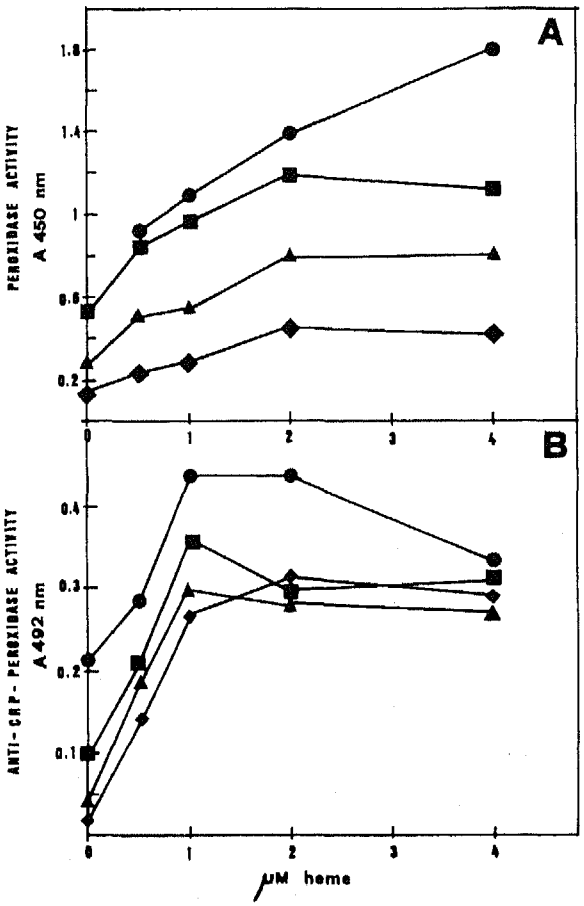

Fig. 1. Peroxidase activity (*A*) and anti-CRP-peroxidase activity (*B*) of aged conjugated after incubation with increasing amounts of heme. The purified conjugate was aged for 3 months at an Ig concentration of 34 μg/ml (0.22 μM coupled peroxidase) in dilution buffer (see materials and methods section) at 4° C. After diluting (1/5–6.8 μg/ml) in several concentrations (see figure) of sonicated heme-dilution buffer, the samples were incubated at 37° C overnight. Peroxidase activity (*A*) and anti-CRP-peroxidase activity (*B*) were measured as indicated in the materials and methods section. After incubation with heme, conjugates were further diluted with dilution buffer as follows: (●) no dilution, (■) 1/2, (▲) 1/4, and (◆) 1/8, and activity measured. Single point measurements were made.

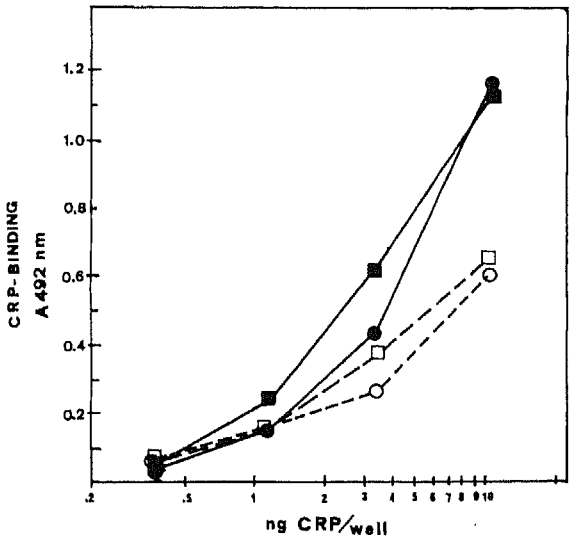

Fig. 2. Activity of aged conjugates in the CRP-binding assay after incubation with or without heme. The purified conjugate was aged for 2 months at an Ig concentration of 34 μg/ml (0.22 μM coupled peroxidase) in dilution buffer at 4° C (see materials and methods section). After diluting to 6.8 μg/ml Ig concentration in dilution buffer with 1.62 μM heme or in dilution buffer without heme, the samples were incubated for 1 and 7 days at 37° C. The assay was performed as indicated in the materials and methods section. Single point measurements were made. Conjugates aged in dilution buffer were further incubated in dilution buffer (O------O, □-----□) or in dilution buffer with 1.62 μM heme (●——●, ■——■). Incubation at 37° C for 1 (O, ●) or 7 (■, □) days.

ent amounts of CRP. No significant differences could be demonstrated in the activities of the conjugates incubated with heme at 1 or 7 days. In both cases the response was proportional to the CRP concentration. The response obtained with conjugates incubated with heme showed an almost two-fold increase over the response obtained with conjugates incubated without heme. This relative increase varied between 1.8–3-fold depending on the conjugate preparation, time at 4° C, buffer and final dilution after the Sephadex G-200 step.

When conjugates were stored at Ig concentrations of 20.4 mg/ml or 3.4 mg/ml in 1% BSA, 50% PBS pH 7.4 and 50% glycerol at −20° C for 2–6 months or at 34 mg/ml in PBS at 4° C for 4–5 months, the presence of heme in the storage buffer made no difference to the final activity of the conjugates. When 0.5 μM heme was added to conjugates diluted to an Ig concentration of 68 μg/ml in dilution buffer and stored at 4° C for 2 months the activity in a CRP-binding assay was 1.2-fold higher than that of conjugates stored at 3.4 mg/ml heme at −20° C. The same conjugates kept for a further 6 months at 4° C and 68 μg/ml in dilution buffer in the presence or in the absence of added heme showed that both peroxidase activity and anti-CRP-peroxidase activity were 1.4-fold higher in conjugates stored with heme than in those without it (Table I).

TABLE I

ACTIVITY OF CONJUGATES STORED AT DIFFERENT IMMUNOGLOBULIN CONCENTRATIONS AND DIFFERENT TEMPERATURES WITH OR WITHOUT HEME

Ig (μg/ml)	Storage (° C)	Storage (months)	Presence of heme	Activity A_{492nm}
3400	−20	2	−	1.17 ± 0.035 (2)
68	4	6	−	0.87 ± 0.014 (2)
68	4	2	+	1.42 ± 0.029 (4)
68	4	8	+	1.22 ± 0.14 (2)

Key: Conjugates were stored at −4° C in dilution buffer (see materials and methods section) in the absence or in the presence of 0.5 μM heme. Control conjugates were stored at −20° C in 1% BSA, 50% PBS, 50% glycerol in the absence of heme. Activity was measured by the CRP-binding assay as described in the materials and methods section at 160 ng CRP/ml. After conjugation, aliquots were stored at 3.4 mg/ml Ig concentration at −20° C in the absence of heme and at 68 μg/ml (0.44 μM peroxidase) at 4° C in the presence of 0.5 μM sonicated heme. After 2 months conjugates were assayed at 6.8 μg/ml and were further stored at 68 μg/ml at 4° C in the absence or in the presence of heme (0.5 μM). After 6 months conjugates were assayed at 6.8 μg/ml. Averages and SD are given. The number of replicates are in parentheses.

Discussion

Some of the practical problems associated with keeping a few microliters of immunoglobulin conjugate at 6.8 mg/ml include buffer evaporation, the necessity of using small containers, the difficulty of processing on a large scale, the need of reconstitution steps, and low reproducibility.

Activity losses of 50–74% were found in experiments designed to store purified conjugates in higher volumes and at more flexible concentrations (about 68 μg/ml). Purified peroxidase is

quite stable as lyophilized dry powder. Even 25 μM aqueous solution maintains its activity undiminished for over a year at 5°C (data not shown). However after conjugation to the antibody and purification, the concentration of peroxidase in a conjugate at 340 μg/ml was 2.2 μM. A further 5–10-fold dilution was necessary for optimal usage. Under these conditions, it was theoretically possible that loss of heme could be lowering the activity in the conjugate and that adding heme back could restore some of the activity lost.

Since hemoglobin contains the same prosthetic group as peroxidase and could be easily obtained, it was used as a source for heme. Aged conjugates were either incubated overnight or stored for prolonged intervals in the absence or the presence of heme and the effect on activity measured. These experiments showed, firstly, that the addition and overnight incubation with ≥ 1 μM heme to aged antibody-peroxidase conjugates kept at high dilutions, increased the peroxidase activity to a maximum of 2–3-fold; secondly that the increase was not due to the added heme, since heme alone did not show peroxidase activity and activity measured by binding to CRP-coated wells (Fig. 1*B*) or CRP-binding assay (Fig. 2) also showed the above mentioned increase, and, thirdly, that inclusion of 0.5 μM heme in the storage buffer of conjugates preserved the peroxidase activity more efficiently than similar conjugates kept without heme (after 6 months of storage at 4°C highly diluted).

For convenience a 0.5 μM concentration of heme was used for highly diluted, 4°C storage experiments (Table I). This minimal concentration of heme is about the same concentration of heme as is bound to the antibody-coupled peroxidase (0.44 μM heme-peroxidase at 68 μg/ml). It seemed to be enough to recover 120% of the activity with respect to the highly concentrated conjugates kept at −20°C for 2 months. In contrast under the same conditions but in the absence of heme a 26% loss of activity was observed (Table I).

To increase volumes and dilution one could allow the free peroxidase to remain in the storage buffer, but at least in the CRP-binding assay, the elimination of free peroxidase by Sephadex G-200 chromatography permits a reduction in background level from 10% to 2% of the maximal signal (data not shown). Lyophylization also presents problems because of the low volumes, reconstitution steps, standardization from batch to batch and non-reproducible loss of activity (data not shown). The present procedure increases the stability of diluted conjugates used in this study but further work is necessary to determine its relevance to other systems and to characterize the effect fully.

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