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Decrease of natural antiphosphorylethanolamine antibodies by injection of C-reactive protein

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1. Summary

Natural levels of goat anti-phosphorylethanolamine (anti-PE) antibodies decreased after injection of purified human C-reactive protein (CRP). The concentration of anti-CRP antibodies followed a continuous increment from non-detectable levels to a plateau at 80 days after first injection.

2. Introduction

C-reactive protein (CRP) is a human plasma protein rising in concentration after tissue injury, infection or inflammation, and returning to normal level as recovery occurs. The CRP molecule has a molecular weight of about 115000. It consists of 5 identical non-glycosylated polypeptide monomers, non-covalently associated in a disc-like configuration with cyclic pentameric symmetry and containing one internal disulphide bridge per monomer. CRP undergoes calcium-dependent binding to phosphorylamine compounds such as phosphorylcholine, PC and phosphorylethanolamine, PE [1].

On the other hand, lymphocytes making antibodies against PC are known to exist in non-immunized young mice and binding sites for PC have been identified in these anti-PC antibodies [2]. The existence of anti-PC antibodies has been related to the fact that phosphorylamines are abundant environmental antigens [3].

This report shows that when goats are immunized with human CRP the ensuing anti-CRP antibody response is simultaneous with a reduction of the level of natural antibodies to PE.

Key words: Antiphosphorylethanolamine antibodies; C-reactive protein; Natural antibodies

Abbreviations: CRP, C-reactive protein; PE, phosphorylethanolamine.

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3. Materials and Methods

3.1. *CRP purification*

The method of Pontet et al. [4], modified by Martinez and Coll [5], was used to purify CRP. CRP concentrations were calculated from A280 nm and ϵ 1%, 19.5. Immunodiffusion was carried out in 200 mM borate, 75 mM NaCl, 2.5 mM $CaCl_2$, 1% agar, 100 mg/l Merthiolate, pH 8. Polyacrylamide gel electrophoresis in the presence of 0.1% SDS and β-mercaptoethanol was used in determining purity. Only one single Coomassie staining band was visualized, at about 21 kDa [5].

3.2. *Immunization protocols*

Two goats of about 20–30 kg weight were injected intramuscularly in the legs in 4 different places. A total of 1 mg of purified CRP in 5 ml of 50 mM sodium phosphate, pH 7.4, mixed with 5 ml of Freund's adjuvant were given each day. Complete Freund's was used in the first injection and then incomplete Freund's for the rest. The injections were given in two sets of 5 weekly injections at 0 to 50 days

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and then at 120 to 160 days. Animals were bled periodically by the jugular vein. About 40–50 ml of blood was taken.

The blood was allowed to clot and serum was obtained by centrifugation and frozen at −20 °C until use. After 200 days all the serum taken was pooled and frozen at −20 °C until use.

3.3. *Purification of anti-PE antibodies*

To purify anti-PE antibodies, about 50 ml of pooled goat antiserum to human CRP were dialyzed against 20 mM Tris, 0.1 M NaCl, 10 mM $CaCl_2$, pH 8 (TNC buffer) and then passed through a column of agarose immobilized 2-aminoethyl dihydrogen phosphate (Pierce, Illinois, USA). After washing the column with 10 volumes of the dialysis buffer described above, the retained material was firstly eluted by chelating calcium with 20 mM Tris, 0.1 M NaCl, 20 mM citrate, pH 8, and secondly by 50 mM ethylenediamine, pH 11, (in this latter case fractions of 1 ml were collected in 50 µl 2 M Tris, pH 4). Two peaks of absorbance at 280 nm were obtained, one for each elution buffer used. After elution by chelating calcium the pooled fractions of the protein peak were identified as goat CRP by immunodiffusion against anti-CRP antibodies (Biomerieux, France) and by CRP assay in solid-phase PE (as will be described later). After elution with ethylenediamine the pooled fractions of the protein peak were identified as goat anti-PE antibodies by immunodiffusion against rabbit anti-goat IgG (Nordic, Tilburg, The Netherlands) and by anti-PE assay in solid-phase PE (as will be described later). Polyacrylamide gel electrophoresis in the presence of 0.1% SDS and β-mercaptoethanol detected major Coomassie staining bands at about 70 and 25 kDa.

3.4. *Anti-PE antibody binding to solid-phase PE*

Solid-phase PE was prepared as described by Martinez and Coll [5, 6]. Briefly, Microwell module F-16 medium binding capacity plates (Nunc, Kamstrup, Denmark) were coated with polylysine (Sigma Chemical Co, St Louis, MO) dried and then incubated overnight with orthophosphorylethanolamine (Sigma) and 1% glutaraldehyde in 10 mM sodium phosphate, 150 mM NaCl, pH 7.4. After washing, the plates were kept vacuum-sealed at 4 °C until use.

To perform the assay, samples were diluted 200-fold in dilution buffer (0.2 M sodium borate, 75 mM NaCl, 2 mM $CaCl_2$, 0.1% Merthiolate, 1% bovine serum albumin, 0.05% Tween 20, pH 8), added to the wells and incubated for 60 min at room temperature. After the plates were washed once in 10-fold diluted dilution buffer they were incubated with rabbit anti-goat IgG (H+L) conjugated to peroxidase (Nordic), in dilution buffer for 30 min at room temperature. After washing, 50 µl of 150 mM citrate buffer with 3 mM H_2O_2 and 1 mg/ml of orthophenylenediamine was added and colour development stopped with H_2SO_4 4 N after 30 minutes. The wells were measured in a SLT EAR 400 FW spectrophotometer at 492–620 nm (Pasteur, Marnes-La-Coquette, France). The absorbance at 620 nm was used to correct for individual non-significant differences between wells. Results were calculated by averaging two independent assays each by duplicate.

A calibration curve was constructed by the use of goat anti-PE antibodies purified by affinity chromatography as described above. Intra-assay coefficient of variation was about 10 percent and linearity was from 1 to 30 µg/ml of anti-PE. The equation of the line was $y = 0.43, \ln x + 0.21$ ($r = 0.997$, $n = 5$) where y was the absorbance at 492–620 nm and x was the anti-PE concentration in µg/ml.

3.5. *Anti-CRP antibody binding to solid-phase CRP*

Goat anti-CRP antibodies were measured on CRP-coated microtiter wells as described by Iturralde and Coll [7] and Coll [8]. A calibration curve was constructed by the use of goat anti-CRP antibodies purified by affinity chromatography on CRP-Sepharose (see below). The equation of the line was $y = 0.35 \ln x + 0.94$ ($r = 0.996$, $n = 6$), where y was the absorbance at 492–620 nm and x was the anti-CRP concentration in µg/ml.

3.6. *Separation of anti-CRP antibodies by PE saturated CRP-affinity column*

Ten ml of goat anti-human CRP were dialyzed against 20 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, pH 8, (TNC buffer) and then passed through a CRP-Sepharose column (10 mg of purified CRP per ml of CNBr activated Sepharose) at 3 ml/h. Bound anti-CRP was eluted with 50 mM ethylenediamine, pH 11, in 1.5 ml fractions over tubes filled out with 50 µl 3 M Tris, pH 5. Twenty mg of anti-CRP were

recovered. After washing the column with the TNC buffer, 15 ml of 0.1 M PE in TNC were passed through the column and incubated overnight, then the column was again washed with TNC and 5 mg of the pooled anti-CRP from the first chromatography, dialyzed against TNC, were passed again through the column. Bound anti-CRP was eluted as above.

4. Results

Anti-PE antibodies were detected in serum from goats, by solid-phase immunoassay using PE-coated plates as described in Section 3. By calibration with anti-PE antibodies purified by affinity chromatography over agarose immobilized 2-aminoethyl dihydrogen phosphate the anti-PE antibody level in normal goats was 9.2 ± 0.4 (n = 8) μg per mg of serum protein. Anti-PE antibody level in commercial goat antiserum against rabbit IgG was 13.6 ± 0.5 (n = 2) μg per mg of serum protein (Table 1).

Table 1
Anti-human CRP and anti-PE antibodies in serum from immunized goats.

	μg/mg of serum protein	
Injected with	anti-human CRP	anti-PE
nothing[a]	ND (6)	9.2 ± 0.4 (8)
rabbit IgG[b]	ND (2)	13.6 ± 0.5 (2)
human CRP[c]	14.0 ± 4 (2)	4.8 ± 0.2 (2)
human CRP[d]	17.1 ± 0.4 (2)	4.1 ± 0.1 (2)
human CRP[e]	20.6 ± 3 (2)	3.8 ± 1.2 (4)
human CRP[f]	24.1 ± 10 (2)	5.1 ± 1.1 (4)
human CRP[g]	16.6	4.5

[a] Equal amounts of sera from 2 uninjected goats was pooled. [b] Antiserum against rabbit IgG from Nordic (Tilburg, The Netherlands). [c] Antiserum against human CRP from Biomerieux (France). [d] Antiserum against human CRP from Difco (Detroit, MI). [e] Antiserum from goat number 1; 124 days after first bleeding (see Section 3). [f] Antiserum from goat number 2; 124 days after first bleeding (see Section 3). [g] Pooled antiserum against human CRP obtained as indicated in Section 3. Sera were assayed by enzyme immunoassay on PE-coated plates, except [g], by affinity chromatography on PE-Sepharose. Results are the mean ± standard deviation and the number in parentheses indicates the number of assays. ND, not detected.

The anti-PE antibody decreased to 3.8 – 5.1 μg per mg of protein in commercial or experimental serum obtained from goats hyperimmunized with human CRP. On the other hand, isolation of anti-PE antibodies by affinity chromatography of pooled hyperimmunized goat serum as described in Section 3 yielded 4.5 μg of anti-PE antibodies per mg of serum.

Next, the time course of induction of anti-human CRP antibodies and evolution of anti-PE antibodies was studied in 2 goats after hyperimmunization with purified human CRP. Figure 1 shows the results of the experiment. Following the first set of human

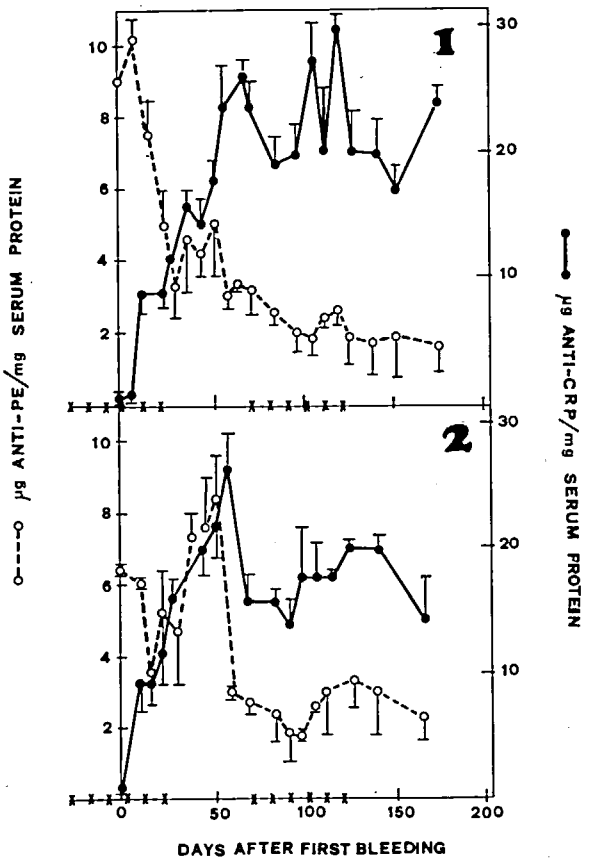

Fig. 1. Anti-PE and anti-human CRP antibody concentration in goats during immunization with human CRP. Animals were bled and sera obtained at the times indicated in the figure. Goat anti-PE (o --- o) and anti-human CRP (● ——— ●) antibodies were assayed as described in Section 3. Results are the mean of two assays and the ranges observed. Goats number 1 and 2 were used. X, time of injections.

CRP injections, levels of anti-CRP showed a continuous increment from non-detectable to a plateau at 20–30 μg of anti-CRP per mg of serum protein in both goats. A 10–20% decrease of levels followed, but it was again recovered after the second set of injections. The concentration of anti-PE antibodies after the first set of human CRP injections followed a decrease from the initial levels either continuous (goat number 1) or with a peak at about 45 days (goat number 2). At about 150 days after first bleeding, final anti-PE concentrations were 5.6 (goat 1) or 2.9 (goat 2)-fold lower than the initial.

These results suggested some relationship between the PE binding sites of human CRP and of goat anti-PE antibodies. An estimation of the extent of antigenicity to goats of the PE binding site of human CRP was obtained by fractionation of goat polyclonal anti-CRP in a Separose-CRP column. Anti-CRP antiserum when passed through the column lost its anti-CRP activity. Anti-CRP was eluted from the column with a yield of 2 mg/ml of antiserum. All the anti-CRP activity was IgG as indicated by reducing polyacrylamide gel electrophoresis (data not shown). When this purified anti-CRP IgG was passed again through the same column after being saturated with PE, 80% of the anti-CRP activity was not bound to the column. An estimation of antigenic relationship between human CRP and goat anti-PE, was made by immunodiffusion against goat anti-human CRP (all at 1 mg of protein per ml). Human CRP gave a strong band of immunoprecipitation but goat anti-PE did not show any band (not shown).

5. Discussion

Levels of anti-human CRP in control goats were negligible. In contrast, levels of anti-PE were significant (Table 1). Phosphorylcholine (PC) is an abundant environmental antigen; the serum of young mice has an unusually high level of anti-PC antibodies, which level increases with age, and high anti-PC antibodies levels are also present even in germ-free animals [3]. Since the chemical structures of PC and PE are very similar, it was not unexpected to find high levels of anti-PE antibodies in control goats. Hyperimmunization with human CRP but not with other unrelated antigen (rabbit IgG) seem to be correlated with decreased levels of anti-PE antibodies (Table 1). These initial observations were confirmed by time course experiments in 2 goats. The behaviour of anti-human CRP (increasing) and anti-PE antibodies (decreasing) was consistent with the idea that both levels were interrelated (Fig. 1). Similar observations were made in 5 rabbits injected with human CRP (not shown).

According to the frequencies of monoclonal antibodies against human CRP [9, 10] and to the inhibition by PE of the goat anti-CRP binding to CRP affinity columns, one of the most important epitopes of CRP seems to contain the phosphorylamine binding site. Therefore injected CRP, induced anti-CRP, and natural anti-PE, could possibly interact because of their structural similarities and/or complementarities. The failure to detect any cross-reactivity by immunodiffusion between goat antibodies specific for human CRP and natural anti-PE antibodies with goat anti-CRP could be due to a different structure of the PE binding site of the human CRP and the natural anti-PE antibodies, or to a low concentration of the goat anti-PE binding site antibodies that would cross-react with its own natural anti-PE antibodies.

References

[1] Gotschlich, E. C. and Edelman, G. M. (1965) Proc. Natl. Acad. Sci. USA. 54, 558–566.
[2] Lai, E. H. C., Kabat, E. A., Meienhofer, J., Heimer, E. P., Olson, A. J. and Lerner, R. (1987) Nature 325, 168–171.
[3] Zarhary, D. and Klinman, N. R. (1986). J. Immunol. 126, 368–370.
[4] Pontet, M., Ayrault-Jarrier, M., Burdin, J., Genlin, M. and Engler, R. (1979) Biochemie 61, 1293–1299.
[5] Martinez, J. and Coll, J. (1987) Clin. Chem. 33, 2185–2190.
[6] Coll, J. M. (1987) J. Immunol. Methods. 104, 259–263.
[7] Iturralde, M. and Coll, J. (1984) Rev. Esp. Fisiol. 40, 279–288.
[8] Coll, J. M. (1987) J. Immunol. Methods, 104, 219–222.
[9] Martinez, J. and Coll, J. (1987) Clin. Chim. Acta (in press).
[10] Kilpatrick, J. M., Kearney, J. F. and Volanakis, J. E. (1982) Mol. Immunol. 19, 1159–1165.