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Isolation and characterization of C-reactive protein from the dog

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Summary. Using calcium-dependent affinity chromatography on Sepharose-bearing, covalently-coupled pneumococcal C-polysaccharide, a protein was isolated from the serum of dogs that had undergone general anaesthesia and major surgery. This protein was confirmed as the canine analogue of C-reactive protein (CRP) in other species by virtue of its electron microscopic appearance, subunit composition and behaviour as an acute phase reactant. Dog CRP had an apparent molecular weight of approximately 100,000 and was composed of five subunits of approximately 20,000 MW each. Two of the five subunits in each molecule were glycosylated. Negatively stained preparations had the typical cyclic pentameric disc-like structure of proteins of the pentraxin family, and in some preparations had a tendency to form stacks. Serum from normal healthy dogs of various strains usually contained less than 5 mg/l of CRP but, following the stimulus of major surgery, an increase in the CRP concentration was first detected at 4 hr.

Abbreviations: CRP, C-reactive protein; EDTA, ethylenediaminetetra-acetic acid sodium; IEF, isoelectric focusing; M_r, relative molecular mass; PAGE, polyacrylamide gel electrophoresis; PAS, periodic acid-Schiff; SAP, serum amyloid P component; SDS, sodium dodecyl sulphate.

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INTRODUCTION

C-reactive protein (CRP) is the classical acute phase plasma protein of man, and related proteins have long been known to exist in the sera of other mammals. This was first demonstrated in the monkey and rabbit using cross-reactivity with anti-CRP antisera (Gotschlich & Stetson, 1960) and by the capacity of the CRP in these species to react with pneumococcal C-polysaccharide (Abernethy, 1937; Anderson & McCarty, 1951). Subsequently, CRP was demonstrated in the dog by its cross-reactivity in a latex agglutination test for human CRP and shown to behave as an acute phase reactant with kinetics similar to those of human CRP (Dillman & Coles, 1966). Dog CRP was isolated and specific antisera developed for its quantitation (Riley & Zontine, 1972) but no detailed biochemical characterization was undertaken.

There has recently been a resurgence of interest in CRP for two reasons. Firstly, it is now known that proteins displaying major amino acid sequence homology, similar molecular configuration and calcium-dependent ligand binding properties are present in many vertebrate sera, and even in some invertebrates (Baltz *et al.*, 1982). These proteins include CRP-like molecules and others related to human serum amyloid P component (SAP), which itself shares 70% identity of sequence with human CRP. The whole family of

molecules are called pentraxins (Osmand *et al.*, 1977) on account of their pentameric annular appearance in the electron microscope. Secondly, with the advent of precise, rapid immunochemical assays for human serum CRP and their application to patients with a wide range of different diseases, it has been established that routine application of this measurement can make an important contribution to patient management (Pepys, 1982).

As part of our continuing programme of investigation of the phylogeny of the pentraxins, we have isolated and undertaken a preliminary characterization of CRP in the dog and we report the results here. Furthermore, we have raised anti-dog CRP antiserum, established an assay for dog CRP and applied it to the formal demonstration of the behaviour of this protein as an acute phase reactant.

MATERIALS AND METHODS

Dog sera

Blood for the isolation of CRP was obtained from mongrel, collie and greyhound dogs 6–8 hr after the onset of surgery under general anaesthesia. After clotting at 4° overnight, serum was separated and stored at −20°.

Isolation of CRP

CRP was isolated from pools of about 500 ml of acute phase dog serum by the same calcium-dependent affinity chromatography procedures previously used for CRP from man and other species (Pepys, Dash & Ashley, 1977; Pepys *et al.*, 1978; de Beer & Pepys, 1982). After elution from a column of pneumococcal C-polysaccharide coupled to cyanogen bromide-activated Sepharose (Pharmacia [G.B.] Ltd, Milton Keynes) the protein was further purified by gel filtration on a 1·6 × 84 cm column of Sephacryl S-300 (Pharmacia) eluted at 6 ml/hr with 0·01 M Tris, 0·14 M NaCl, 0·01 M EDTA, 0·1% w/v azide, pH 8·0 (Tris-saline-EDTA).

Estimation of molecular weight

The apparent relative molecular mass (M_r) of isolated dog CRP was estimated by gel filtration chromatography on the same Sephacryl S-300 column as above, which had been calibrated with standard globular proteins of known molecular weight and with isolated human CRP (de Beer & Pepys, 1982). Sedimentation equilibrium studies were performed on a Beckman model E analytical ultracentrifuge according to the meniscus depletion method of Yphantis (1964). The concentrations, buffers and rotor speeds used are shown in Table 1.

Table 1. Analytical ultracentrifugation of dog CRP

Dog CRP in Tris/NaCl/EDTA buffer

Concentration (mg/ml)	Speed (r.p.m.)	MW
0·24	14,000	118,500
0·24	17,000	115,600
0·47	14,000	113,800
0·47	17,000	109,800
0.70	14,000	117,700
0·70	17,000	114,600
	Average	115,000

Dog CRP in 6·00 M guanidinium chloride

Concentration (mg/ml)	Speed (r.p.m.)	MW
0·23	44,000	21,000
0·23	48,000	20,000
0·35	44,000	17,800
0·35	48,000	18,200
0·70	44,000	18,800
0·70	48,000	19,200
	Average	19,200

Polyacrylamide gel electrophoresis (PAGE)

PAGE analysis of dog CRP was undertaken with the native, undenatured protein in 4–30% w/v gradient gels (Pharmacia) run according to the manufacturer's instructions. Sodium dodecyl sulphate (SDS)-10% w/v PAGE was run according to the method of Laemmli (1970) and calibrated with marker proteins of known M_r (Pharmacia) and with isolated human CRP and SAP (de Beer & Pepys, 1982). In addition to conventional protein staining with Coomassie Blue (R250), glycoproteins were stained with periodic acid-Schiff stain (PAS) (Glossman & Neville, 1971).

Identification of dog CRP as a glycoprotein

In addition to the PAS staining of SDS-PAGE, dog CRP (4·7 mg) was confirmed as a glycoprotein by its complete uptake onto a 10 ml column of Concanavalin A-Sepharose (Pharmacia) in 0·01 M Tris, 0·14 M NaCl, 0·002 M $CaCl_2$, 0·1% w/v azide pH 8·0 (Tris-saline-Ca). It was then quantitatively recovered by elution with 0·5 M α-methyl mannoside (Sigma London Chemical Co. Ltd, Poole, Dorset) in the same buffer.

Isoelectric focusing (IEF)
Dog CRP was subjected to IEF in agarose gel using the reagents and methods specified by the manufacturer (Pharmacia) and using the LKB Multiphor apparatus (LKB Instruments Ltd, South Croydon, Surrey). Marker proteins of known pH (between 3–10) (Pharmacia) were run as calibrators and the pH gradient was also measured directly with a surface electrode (Corning Medical, Halstead, Essex).

Electron microscopy
Dog CRP preparations were examined by the negative staining method as described elsewhere (de Beer *et al.*, 1982).

Antisera
A rabbit was immunized by injection of isolated dog CRP in complete Freund's adjuvant and boosted by subsequent injections in incomplete adjuvant. The antiserum was tested by double immunodiffusion and immunoelectrophoresis in agarose gels (1% w/v in 0·075 M veronal buffer containing 0·01 M EDTA at pH 8·6). Antisera directed against pentraxins of other species were available in the laboratory (Pepys *et al.*, 1977; 1979; 1982; de Beer *et al.*, 1982) and were tested against dog CRP preparations by immunodiffusion.

Assay for dog CRP
A standard electroimmunoassay (Laurell, 1972) for dog CRP was established using rabbit anti-dog CRP serum and the same conditions reported elsewhere for electroimmunoassay of human CRP (Fagan *et al.*, 1982). The assay was calibrated with standards of isolated pure dog CRP.

RESULTS

Isolation of dog CRP

Application to acute phase dog serum of the same calcium-dependent affinity chromatography procedures which have been used to isolate human, rabbit, rat and plaice CRP, yielded a protein preparation which was further purified by gel filtration. This procedure separated high molecular weight material from the well-defined symmetrical peak of CRP (Fig. 1). When antiserum to the dog CRP had been produced and an assay for it established, it was determined that the final recovery of the protein was approximately 60% of the original quantity in the acute phase serum.

Characterization of dog CRP in PAGE

Non-denatured samples of dog CRP run in 4–30% gradient PAGE consistently failed to produce any clearly defined bands, but instead yielded a smear of stained material extending through most of the gel. In SDS-PAGE (Fig. 2) all preparations gave two distinct closely spaced bands, the faster migrating of which comprised 60% of the total protein by scanning densitometry. In reduced, as compared with non-

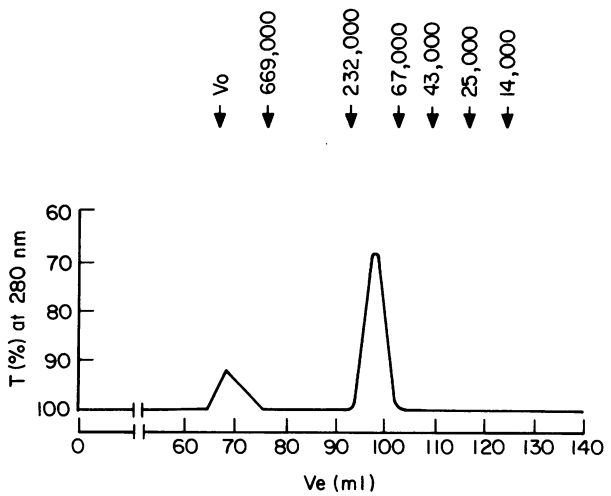

Figure 1. Gel filtration of dog CRP on Sephacryl S-300. Arrows indicate the elution volume of globular marker proteins.

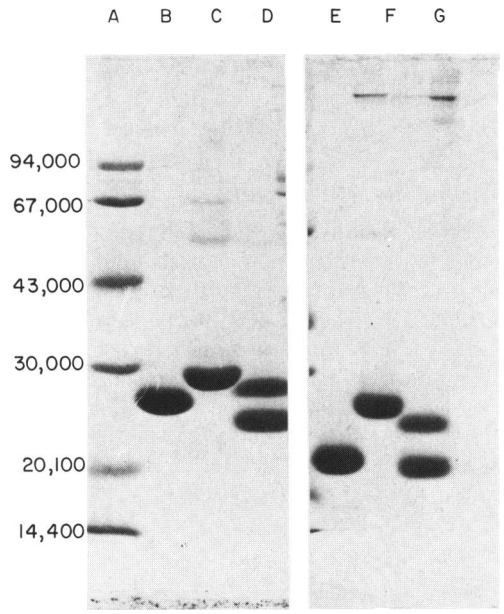

Figure 2. SDS-10% PAGE of dog CRP. Lane A, marker proteins of known molecular weight; B, human CRP; C, human SAP; D, dog CRP. Lanes E-G, unreduced human CRP, SAP and dog CRP respectively.

reduced, samples these bands migrated less far into the gel, indicating that both these subunits contained intrachain disulphide bonds. The apparent M_r of the two subunits after reduction, determined according to a calibration curve derived from standards of known M_r, was 28,600 and 24,600. In the non-reduced state, they migrated with apparent M_r of 25,700 and 20,400 respectively. When the calibration curve was corrected for the anomalous behaviour of human CRP in SDS-PAGE (Pepys *et al.*, 1978) the apparent values for the dog CRP subunits were: reduced, 23,550 and 19,400; non-reduced, 20,200 and 16,200.

Estimation of molecular weight of dog CRP

Samples of dog CRP eluted from Sephacryl S-300 gel filtration columns as a single symmetrical peak corresponded to an apparent M_r of 102,300. Correction of this value in accordance with the known anomalous behaviour of human CRP on gel filtration gave a figure of 91,200. Sedimentation equilibrium analysis at various speeds at different concentrations of dog CRP in Tris-saline-EDTA gave a mean value for M_r of 115,000, whilst in 6·0 M guanidine, the subunits gave a mean result of 19,200 (Table 1). The relatively wider range observed after dissociation (coefficient of variation 6·2% versus 2·7% without denaturation) is compatible with the presence of two differently sized subunits as indicated in SDS-PAGE.

Electron microscopy of dog CRP

The molecules appeared as annular pentameric discs composed of subunits, with a tendency for the discs to stack face-to-face. They closely resembled pentraxins of all other species examined by this method (Fig. 3).

Isoelectric focusing of dog CRP

Samples of isolated dog CRP gave a series of 12 closely spaced bands in the range of pH 5·60–6·65, and a further single band at pH 5·3 (Fig. 4).

Characterization of dog CRP as a glycoprotein

When preparations of isolated dog CRP were passed over a column of Con A-Sepharose, all the protein was retained and could then be eluted with α-methyl mannoside, but not with Tris-saline-EDTA.

Application of the PAS stain to dog CRP run on an SDS-PAGE revealed positive staining only of the higher molecular weight subunit. Controls run in adjacent lanes were human CRP, which is not glycosylated and did not stain with PAS, and human SAP, a known glycoprotein which did stain.

Production of antiserum to dog CRP

Immunization of a rabbit with isolated dog CRP induced production of an antiserum which precipitated in gel with the CRP, but also reacted weakly with an additional antigen present in both normal and acute phase dog serum. In immunoelectrophoresis, this other antigen covered a broad range of β-γ mobility and was presumably immunoglobulin, whilst the CRP itself had slow β mobility.

Various antisera against pentraxins of other species, including rabbit and sheep anti-human CRP, rabbit anti-rat CRP and SAP, rabbit anti-plaice CRP and SAP and rabbit anti-mouse SAP, all failed to cross-react with acute phase dog serum or isolated dog CRP. Dog CRP also did not react in a commercial latex agglutination test for human CRP using sheep anti-

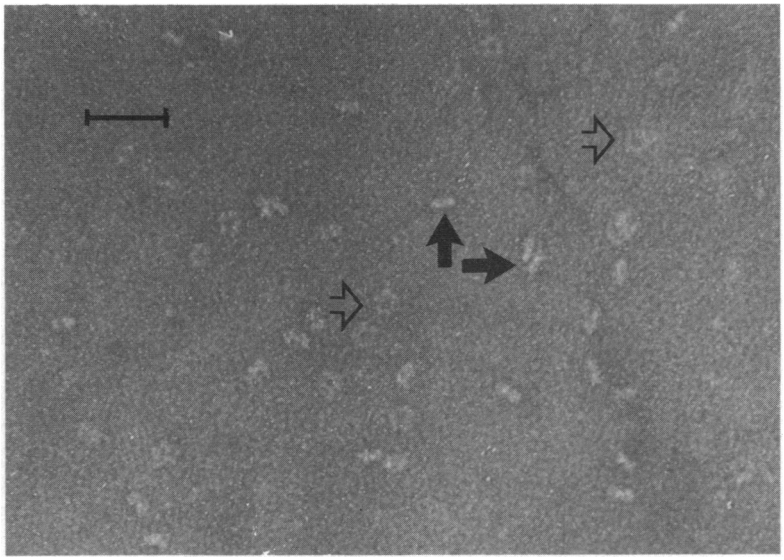

Figure 3. Electron micrograph of dog CRP, side on (black arrow) and face on (open arrow). Bar represents 30 nm.

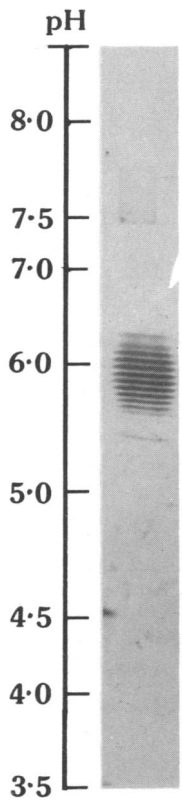

Figure 4. Isoelectric focusing of dog CRP in agarose gel.

human CRP (CRP Wellcotest, Wellcome Diagnostics, Dartford, Kent).

Serum concentration of dog CRP

An electroimmunoassay for dog CRP was established using the antiserum described above. The lower limit of sensitivity was about 5 mg/l, the upper limit about 100 mg/l, and the within and between assay coefficients of variation were less than 10%. The weak non-CRP precipitation which extended both anodally and cathodally from the well was easily distinguishable from the sharp CRP rocket.

Using this assay, CRP was not detected in sera from 20 clinically healthy dogs of different age, sex and breed. In five dogs undergoing surgery, serum CRP level at operation was at or below the sensitivity limit of the assay. In these dogs, there was a rapid and significant rise in CRP concentration, first detectable at 4 hr (Fig. 5). Among five animals in which the serum CRP was already raised at the time of operation (range 22–66 mg/l), there was no further increase at 4 hr in three dogs. In the remaining two animals, increases in CRP level were seen at 4 hr (66 to 87 and 24 to 36 mg/l respectively).

DISCUSSION

We have confirmed earlier work which, although not

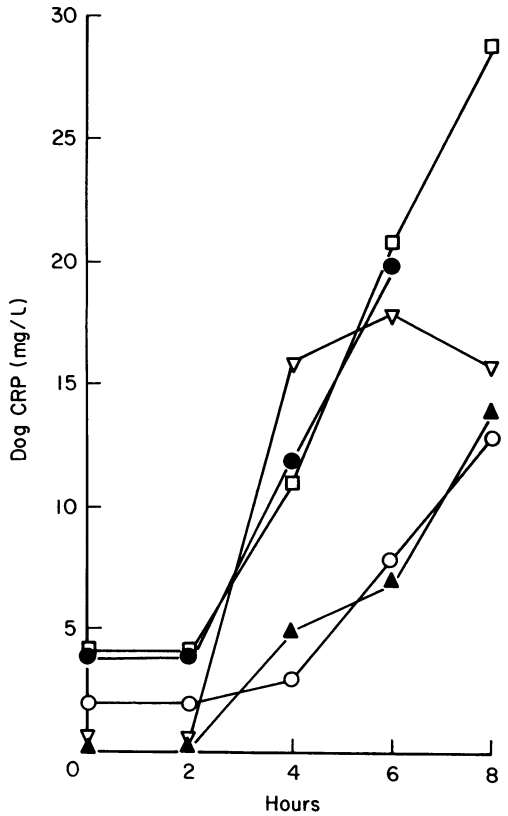

Figure 5. Serum concentrations of CRP during surgery, in five dogs with low starting levels.

involving any appreciable degree of biochemical characterization, demonstrated that dog CRP closely resembles CRP of man, monkey and rabbit in its binding specificity, molecular size and behaviour as an acute phase protein (Riley & Zontine, 1972). In addition, we show here for the first time that dog CRP is a glycoprotein but that only some of its subunits are glycosylated. Our estimates of molecular weight by various methods, taken together with the molecular appearance in the electron microscope, indicate that dog CRP consists of five non-covalently associated subunits, of which two in each molecule are glycosylated whilst the remainder are not. This rather curious situation, and the appearance of multiple closely-spaced bands on isoelectric focusing, have been observed previously in other pentraxins, and there is no doubt, even in the absence of amino acid sequence data, that the present material is a member of this same protein family (Baltz *et al.*, 1982).

Antigenic cross-reactivity between human CRP and dog CRP was reported by earlier workers but we were not able to detect any cross-reactivity with an extensive panel of antisera against pentraxins of man and various species. We have commented elsewhere on the relative rarity of antigenic cross-reaction between pentraxins of different species (Baltz *et al.*, 1982) and the present finding is therefore not surprising.

Perhaps the most notable feature of dog CRP reported here is the speed of its appearance and increase in concentration in the serum following an acute stimulus. Significant elevation was present only 4 hr after an acute stimulus, a more rapid response than has been seen with either human CRP or mouse SAP, another prominent acute phase protein. This augurs well for the clinical veterinary application of assays for dog CRP. By analogy with the clinical value of measurements of human serum CRP and in confirmation of the earlier findings with semi-quantitative tests for dog CRP, it seems likely that precise assays for dog CRP may now find broad general application in canine veterinary practice. This probability is, in fact, borne out by our own recent work, to be published shortly, in which we have observed that serum levels of dog CRP closely reflect the extent of tissue damage and inflammation following trauma, and in a number of infective and non-infective conditions.

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