Studies on canine C-reactive protein

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Canine C-reactive protein (CRP) was isolated and purified. The physicochemical and biological properties were investigated. A simple method for detection was presented.

CRP was first discovered in human serum. It dates back to the study to Tillett and Francis (1930) on capsular polysaccharides of *Diplococcus pneumoniae* in which a substance (CRP) reactive to C-polysaccharides was found in serum from patients with an initial stage of pneumonia but was then misunderstood to be a specific antibody to C-polysaccharides.

Human CRP was first isolated in 1941 by McLeod and Avery. Volanakis and Kaplan (1970) identified choline phosphate of C-polysaccharides to be the major determinant (binding site) of CRP. On the other hand, CRP was reported to rapidly increase in serum after surgical operations or during tissue destruction and acute inflammation in various diseases such as infection, rheumatoid arthritis, myocardial infarction and malignant tumor but to rapidly disappear on recovery from these diseases. This stimulated studies on clinical significance of CRP.

At present, CRP is known to be synthesized in hepatic cells, exist in a very small amount of approximately 0.580 µg/ml (580 ng/ml) in normal human serum and in the event of inflammation or tissue destruction, to rapidly increase within a few hours even to 1,000 times the level that existed in a normal state. This rapidly synthesizing CRP has also
been confirmed to have the half life of 4 - 6 hours.

Since CRP reflects precisely the degree of inflammation and tissue destruction, CRP has been clinically used as an index for evaluation of the activity, development and prognosis of diseases. Today CRP is an essential item to be examined in the diagnosis of diseases which accompany a CRP increase.

The presence of CRP in dogs has been suggested implicitly. However, nothing is known of the details. Basic studies on the true nature of canine CRP may provide an important key to approaches to clinical application.

The author confirmed the existence of CRP in dogs by isolation and purification to investigate the physicochemical and biological properties of canine CRP. A simple method of CRP detection in dogs was also studied. The results are outlined as under.

1. Existence of CRP in dogs

Acute inflammation was induced in Beagle dogs by intraperitoneal inoculation of $1.6 \times 10^{11}$ Staphylococci aureus (2 ml). Rabbits were immunized with an cathodal protein component obtained by block-zone electrophoresis of the dog serum. A rabbit antiserum was thus obtained. By absorption of this antiserum into normal canine serum, the author obtained an antiserum that reacts specifically with a protein having the
mobility hitherto unknown. This antiserum did not react to normal canine serum but only to serum from dogs with acute inflammation induced by various methods. Immuno-electrophoresis of this protein produced exactly the same slow γ-type mobility and electrophoretic pattern as human γ-type CRP. This adequately suggested the identity of this protein to be canine CRP.

II. Purification of canine CRP and preparation of specific antiserum

1. Purification of canine CRP by chromatographic and other procedures

Canine acute phase serum was fractionated by DEAE-Sephacel. A fraction containing CRP was isolated from the first peak by gradient elution using 0.01 M Tris-HCl buffer (pH 8.0) and the same buffer containing 0.3 M NaCl. This fraction was further fractionated by DEAE-Sephadex A-50. Stepwise elution with 0.01 M phosphate buffer (pH 7.6) containing 0.10 - 0.25 M NaCl produced a fraction containing CRP as a main component (CRP fraction) in phosphate buffer that contained 0.25 M NaCl. A small amount of IgG that existed in the CRP fraction was then removed by affinity chromatography using protein A-Sepharose CL 4B.

In agar block electrophoresis of this protein, CRP was isolated as a monocomponent by collecting the component at
the cathodal side 1 cm from the wells.

2. Purification of canine CRP using L-α-lecithin

Purification of CRP from canine acute phase serum was attempted basically by the method of Hokama et al., using L-α-lecithin that contained choline phosphate. Approximately 1 mg of CRP was purified as a CRP monocomponent from 40 ml of serum.

3. Verification of purified CRP

Purified CRP by the above-described 2 methods were both confirmed by immunoelectrophoresis and disc electrophoresis to be the monocomponents having the same mobility and electrophoretic pattern.

4. Preparation of specific antiserum to CRP

By immunization of rabbits with these purified CRP as an antigen, specific antisera that reacted only with these CRP from acute phase serum were obtained.

III. Antigenicity of CRP purified by chromatography and L-α-lecithin

The antigenicity and antibody activity were examined by the Ouchterlony's test between the CRP purified by the above 2 methods and the antisera prepared by immunization with these CRP as an antigen. The test identified that these CRP and antisera were exactly the identical antigens (CRP) and antibodies.
IV. Physicochemical properties of canine CRP

The physicochemical properties of canine CRP were examined to compare the peculiar properties of canine CRP with the properties of human CRP.

1. Molecular weight

In Sephacryl S-300 gel filtration of fresh acute phase serum, CRP was eluted in the latter half of the 3rd peak as a substance containing IgG as a main component. The molecular weight of canine CRP was, therefore, estimated to range from 120,000 to 180,000.

(i) Measurement of the molecular weight by gel filtration

The molecular weight of purified CRP was 157,000, as measured by Sephacryl S-300 gel filtration.

(ii) Measurement of the molecular weight by SDS-PAG electrophoresis

The molecular weight of canine CRP was measured by SDS-PAG electrophoresis using the same CRP as used for gel filtration. The electrophoretic pattern showed 2 bands consisting of a fast moving component and a slow moving component. The molecular weights were 70,000 for the former and 85,000 for the latter, amounting in total to 155,000. This molecular weight almost corresponded to the value (157,000) obtained by gel filtration. In this measurement, pretreatment of the sample with a reducer seems to have separated CRP into 2 peptides due to break-down of the S-S
binding and resulted in the appearance of CRP in 2 bands. These measurement results indicate that the molecular weight of canine CRP may be estimated to be 155,000 - 157,000, slightly larger than that of human CRP.

2. Mobility

Immunoelectrophoresis revealed a formation of precipitin line at slow region. This mobility and pattern corresponded to those of human \( \gamma \)-type CRP.

3. Isoelectric point

The isoelectric point of canine CRP as measured by isoelectric focusing in thin layer PAG was 4.75, which was close to that of human CRP (4.82).

4. Thermal resistance

The antigenicity of canine CRP was completely lost by heating for 15 minutes at 70°C. Canine CRP was a protein slightly more thermolabile than human CRP (70°C, 30 min).

5. Comparison of antigenicity between canine and human CRP

In the Ouchterlony's test, anti-human CRP serum (commercial) reacted slightly with canine CRP. However, anti-canine CRP serum showed no reactivity to human CRP. This finding suggested the presence of a determinant having the partly common antigenicity in canine and human CRP. It was presumed that human CRP was structurally more recognizable in immunized animals than canine CRP.

6. Reactivity of CRP to choline phosphate
As earlier described, canine CRP was purified using L-α-lecithin that contained choline phosphate. This suggested that canine CRP also possesses the reactivity to choline phosphate as does human CRP. Therefore, canine CRP is presumed to have reactivity to choline phosphate of pneumococcal C-polysaccharides.

V. Serum CRP concentration in normal dogs

The serum CRP concentration was determined by enzyme immunoassay (EIA) using 20 serum samples obtained from clinically healthy 1- to 3-year old Beagle dogs (10 males and 10 females). The CRP concentration ranged from 0.198 to 0.826 μg/ml (198 - 826 ng/ml) for the average of 0.486 ± 0.170 μg/ml (486 ± 170 ng/ml). The CRP concentration in normal humans is reported to range from 0.068 to 8.200 μg/ml (68 - 8,200 ng/ml) for the average of 0.580 ± 1.340 μg/ml (580 ± 1,340 ng/ml). The CRP concentration in normal dogs was thus approximately close to that in normal humans.

VI. Postsurgical variation of serum CRP concentration

Using 4 female 3-year-old normal Beagle dogs, the surgery-induced variation of serum CRP concentration was examined by single radial immunodiffusion (SRID).

Two dogs each were used for gastrotomy and oophorohysterectomy. CRP was not detected preoperatively in all
the dogs. However, at 6 hours postoperatively, serum CRF was detected in all of them.

In 2 gastrotomized dogs, the CRP concentration rose to the maximum of 305 and 245 µg/ml on the following day of the operation, which decreased to 106 and 74 µg/ml on the 7th postoperative day. On the 10th day postoperatively, it was further reduced to 61 and 48 µg/ml respectively, a decrease to approximately 1/5 of the peak values. On the 20th and 15th postoperative days, CRP could no longer be detected as it could not preoperatively.

In 2 oophorohysterctomized dogs, the CRP concentration rose to the maximum of 221 and 260 µg/ml on the following day of the operation. In the former, it steadily decreased to 60 µg/ml on the 7th postoperative day and to 25 µg/ml on the 10th day, and finally disappeared on the 12th day. In the latter, it began to gradually decrease from the 2nd postoperative day and fell to 105 µg/ml on the 8th day when the suture was extracted. It was, however, followed by a rapid reincrease, reaching 208 µg/ml on the 10th day. This dog was treated with an antibiotic due to mild suppuration of the abdominal wound that occurred at the time of suture extraction. In response to this treatment, the CRP concentration reddecreased sharply and disappeared on the 20th postoperative day.
VII. Simple method for detection of CRP by reversed passive latex agglutination test

Optimum conditions for antibody sensitization in a latex have not yet been established. The author tested various conditions and obtained the following sensitizing condition.

1. Antibody sensitization condition

IgG antibody isolated from anti-canine CRP serum by protein A-Sepharose CL 4B was adsorbed onto a soap-free polystyrene latex of the particle size 0.12μ (latex) and used as an antibody sensitization latex.

On comparing various conditions, the latex concentration of 2%, sensitization temperature of 37°C, sensitization time of 3 hours and antibody of 30 μg/mg latex were found to be the optimum sensitization condition for the antibody used in the experiment.

2. Treatment of sensitization latex for obtaining distinctive agglutination

The sensitization latex with unadsorbed binding sites blocked by BSA was washed by centrifugation at 15,000 rpm for 15 minutes in 0.01 M glycine buffer (pH 7.3) of the specific gravity 1.040. By precipitation, the sensitization latex that showed distinctive agglutination was obtained.

By this centrifugation process, only the antibody-rich, low surface-charge sensitization latex could be separated.
The use of this latex seemed to have permitted very distinct agglutination.

3. Serum CRP concentration and reversed passive latex agglutination test

Using the serum from the dogs surgically treated, the relation between the serum CRP concentration and agglutination of the antibody sensitization latex was examined.

Agglutination was defined to be ++++ for the serum CRP concentration of 149 - 305 μg/ml, +++ for 91 - 137 μg/ml, ++ for 38 - 95 μg/ml, + for 5 - 39 μg/ml and - for below 5 μg/ml. The CRP concentration and the intensity of agglutination were consistently and definitely correlated, with the exception of one ++ case despite the + serum concentration of CRP.

VIII. Conclusion

The physicochemical properties and some of the biological properties of CRP isolated and purified from canine serum were studied. A simple method for detection of canine CRP was also studied. Following are the results obtained.

1. CRP was isolated and purified from canine acute phase serum by either a combination of ion exchange chromatography and block electrophoresis or the procedure using L-α-lecithin. Antiserum specific to canine CRP was also prepared by immunization in rabbits.

2. The molecular weight of canine CRP was 157,000 by column
gel filtration. SDS-PAGE electrophoresis produced 2 component bands having the molecular weights of 85,000 and 70,000 that amounted in total to 155,000. The molecular weight of canine CRP was thus estimated to range from 155,000 to 170,000.

3. It was suggested that a determinant having the common antigenicity is present in human and canine CRP.

4. Canine CRP was a thermolabile protein that completely loses the antigenicity by heating for 15 minutes at 70°C.

5. The isoelectric point of canine CRP was 4.75. By immunoelectrophoresis, it was a protein having slow γ mobility.

6. Canine CRP also reacted with choline phosphate.

7. The serum CRP concentration in 20 normal dogs ranged from 0.198 to 0.826 μg/ml (0.486 ± 0.170 μg/ml).

8. The serum CRP concentration rapidly increased after experimental surgical treatment but disappeared on recovery. In an animal that developed postoperative suppuration, the CRP concentration rapidly reincreased. This phenomenon precisely coincided with the postoperative behavior of human CRP.

9. The intensity of reversed passive latex agglutination varied in parallel with the serum CRP concentration. Reversed passive latex agglutination test was a simple and sensitive method for determination of CRP.