Simple Isolation of Canine C-Reactive Protein (CRP) by Phosphorylcholine (PC) Affinity Chromatography

Hiroshi FUJISE, Hiroshi TAKANAMI, Masaaki YAMAMOTO, Ikuko OHTA, Shizuo YAMAMOTO¹⁾, Tohru FUKASE²⁾, Masaharu NAIKI³⁾, Sumiyuki AKIHAMA²⁾, Eri OGAWA, and Reiji TAKAHASHI

Department of Pathology, School of Veterinary Medicine, ¹⁾Laboratory of Immunology, College of Environmental Health, Azabu University, Sagami-hara, Kanagawa 229, ²⁾1st Department of Biochemistry, Meiji College of Pharmacy, Setagaya-ku, Tokyo 154, and ³⁾Department of Veterinary Science, National Institute of Health, Shinagawa-ku, Tokyo 141, Japan
(Received 23 July 1991/Accepted 4 October 1991)

J. Vet. Med. Sci. 54(1): 165–167, 1992

KEY WORDS: C-reactive protein (CRP), dog, phosphorylcholine.

C-reactive protein (CRP) is one of the serum constituents which has been called an acute phase reactant [13]. Serum CRP concentration has been used as a clinical index of acute inflammation in human, since the concentration rises with infectious disease and/or tissue damage whereas it is trace in normal serum [1, 7, 8]. The existence of CRP in serum was reported in many animals [13], though the amount and/or the occasion for its appearance varied with the animal species or sex [9, 13]. In dog, several reports showed the usefulness of CRP measurement for clinical index [3–6]. However, it has not been used for routine clinical examination, because there is no commercial kit available for canine CRP measurement. Since CRP is usually measured by immunoassay, it has to be purified for preparation of the antisera.

Canine CRP was isolated by using the binding capacity of the CRP to pneumococcal C-polysaccharide in the presence of Ca [2] as well as human and some other animals [11, 12]. One of the present authors earlier isolated an acute phase protein from canine serum using several steps of chromatography and electrophoresis, and indicated it as CRP [17]. However, the procedure was very complicated and the yield was low. Recently, phosphorylcholine (PC), which inhibited the precipitation reaction of CRP with pneumococcal C-polysaccharide [16], was successfully used as the ligand for human CRP instead of pneumococcal C-polysaccharide [15]. However, PC-affinity chromatography was not useful to purify bovine CRP [10]. Hence, we examined whether PC affinity chromatography is available for isolation of canine CRP.

Acute phase sera, in which the CRP concentration might arise, were obtained from 3 mongrel dogs 2 days after surgery. Normal sera were obtained from 2 beagle dogs which were acclimated in the doghouse of Azabu University for more than 1 year. PC derivatives of bovine serum albumin (PC-BSA) immobilized on SH derivatized Toyopearl HW [15] were the kind gift of Dr. Kohda of the Central Research Lab., Oriental Yeast Co. Ltd., Osaka, Japan.

A column (0.8 × 5 cm) packed with Toyopearl HW 65 conjugated with PC-BSA was used for affinity chromatography of CRP. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl and 1 mM CaCl₂, then 20 ml of CRP-induced serum

Fig. 1. Affinity chromatography of acute phase serum from dog on PC-BSA Toyopearl HW 65 column (0.8 × 5 cm). Twenty ml of acute phase serum was applied on the column equilibrated with 10 mM sodium phosphate buffer containing 150 mM NaCl and 1 mM CaCl₂. After sufficient washing with the buffer, binding fraction was eluted with 10 mM sodium buffer, pH 7.2, containing 150 mM NaCl and 1 mM EDTA. (The time point was indicated by the arrow.)

Fig. 2. Gel filtration of PC-fraction on Sephacryl-S 300 column (1 × 76 cm). A 1.5 mg protein was applied on the column equilibrated with 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl and 1 mM EDTA, then the protein was eluted with the buffer. A 1.8 ml fraction was collected in each tube.

was subjected to the column. As shown in Fig. 1, after sufficient washing with the phosphate buffer containing Ca, Ca-dependent PC-binding fraction (PC-fraction) was eluted from the column with 10 mM phosphate buffer pH 7.2 containing 1 mM EDTA. Then, the PC-fraction was concentrated by ultrafiltration and approximately 1.5 mg protein solution was applied to Sephacryl S-300 gel filtration column (1 × 76 cm). As shown in Fig. 2, the column was eluted with 10 mM phosphate buffer, pH 7.2, containing 1 mM EDTA, and a small peak at void volume and a main peak at 7.2 ml after the void volume were detected. Gel permeation chromatography using Cellulofine GCL 2000 column (1 × 60 cm) was carried out to define the molecular weight of the main peak fraction, which was 120 K dalton.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the main peak fraction consists of two different subunits of 21.5 K and 25.8 K daltons as shown in Fig. 3. The larger subunit was stained with periodic acid-Schiff reagent (PAS), indicating that the subunit is glycosylated (Fig. 3). All these characterization data coincided with the data on canine CRP reported by Caspi *et al.* [2].

Immunological characterizations were performed with the main peak fraction. Antisera to PC fraction were prepared by immunizing goat with the fraction with Freund's complete adjuvant. As shown in Fig. 4, by double diffusion test, the antisera react with the acute phase serum by 2 precipitin line, and with the normal serum by one precipitin line. However, after absorption with the normal sera, the precipitin line was found only with the acute phase sera, but not with the normal sera.

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified CRP. The gel was stained with Coomassie Brilliant Blue G250 (1 and 2), or periodic acid-Schiff's reagent (PAS)(3). A molecular weight calibration kit (Oriental Yeast Co., Ltd.) was also applied (1).

The antisera also showed 2 precipitin line with the PC-fraction, but only one line with the fraction after the gel filtration. The antisera to normal sera, which was prepared by immunizing rabbit with the sera, failed to show a precipitin line with the fraction after gel filtration. Thus, the fraction was not a constituent of normal sera.

As shown in Fig. 5, these results were confirmed by immunoelectrophoresis and the fraction migrated slow γ . The contaminations in PC-fraction were supposed to be IgM and L-chain of IgG by the immunoelectrophoresis, and they were removed by the gel filtration (Fig. 5). The antisera to human CRP indicated partial cross-reactivity with the fraction after the gel filtration and canine acute phase sera, but not with the normal sera (data not shown). Again, these results indicated that the fraction was CRP.

Fig. 4. Double immunodiffusion of canine acute phase serum (A), normal canine serum (B), PC-fraction (C) and CRP (D) against the goat anti canine CRP before (1) and after (2) absorption, and rabbit anti normal canine serum (3). The analysis was performed in a 5% agarose plate in 50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl₂, pH 7.5.

Fig. 5. Immuno electrophoresis of canine acute phase serum (A), normal canine serum (B), PC-fraction (C) and CRP (D) against the goat anti canine CRP before (1) and after (2) absorption and rabbit anti normal canine serum (3). The electrophoresis was carried at 40 mV per 5 cm for 50 min in the same plate as in Fig. 4.

In the dog, the affinity column was useful to isolate CRP, and highly purified CRP was obtained by gel filtration after PC affinity chromatography. The recovery of CRP during PC affinity chromatography was 88% and that of gel filtration was 75%. Thus, the final recovery was approximately 65% of the original quantity in the acute phase serum. In the previous methods, the recovery was 60% by the method using pneumococcal C-polysaccharide for the affinity chromatography [2], and it was 15% by combining several chromatographies and electrophoreses [17]. Thus, present method for CRP isolation was sufficient. The canine CRP was measured from 8 $\mu\text{g/ml}$ by single radiation immunodiffusion assay with the goat anti-canine CRP.

The application of the anti human CRP sera to the measurement of canine CRP was as reported earlier [5]. However, our immunology data showed only partial reactivity between the anti-human CRP sera and canine CRP. Actually, by using one of human CRP measurement kit, one-sixth of the actual amount of canine CRP was detected. Therefore, the kit only detected abnormally elevated CRP concentration and could not distinguish normal and abnormally increased level. Thus, it should be careful about the numbers obtained by application of human CRP kit to canine CRP measurement.

REFERENCES

1. Anderson, H. C. and Mccarty, M. 1950. *Am. J. Med.* 8: 445-455.
2. Caspi, D., Balts, M., Snel, F., Gruys, E., Niv, D., Batt, R. M., Munn, E., Buttress, N., and Pepys, M. B. 1984. *Immunology* 53: 307-313.
3. Caspi, D., Snel, F., Batt, R. M., Bennett, D., Rutteman, G., Hartman, E. G., Baltz, M. L., Gruys, E., and Pepys, M. B. 1987. *Am. J. Vet. Res.* 48: 919-921.
4. Conner, J. G. and Eckersall, P. D. 1988. *Res. Vet. Sci.* 45: 107-110.
5. Dillman, R. C. and Coles, E. H. 1966. *Am. J. Vet. Res.* 27: 1769-1775.
6. Eckersall, P. D., Conner, J. G., and Harvie, J. 1991. *Vet. Res. Comm.* 15: 17-24.
7. Fischer, C. L., Gill, C., Forrester, M. G., and Nakamura, R. 1976. *Am. J. Clin. Pathol.* 66: 840-846.
8. Griffin, D. E., Hirsch, R. L., Johnson, R. T., Soriano, I. L. D., Roedenbeck, S., and Vaisberg, A. 1983. *Infect. Immun.* 41: 861-864.
9. Kuschner, I. 1986. *Prot. Biol. Fluid* 34: 259-262.
10. Morimatsu, M., Sakai, H., Yoshimatsu, K., Minowa, O., Yamamoto, S., Yatomi, K., Fujinaga, T., and Naiki, M. 1989. *Jpn. J. Vet. Sci.* 51: 723-732.
11. Pepys, M. B., Dash, A. C., and Ashley, M. J. 1977. *Clin. Exp. Immunol.* 30: 32-37.
12. Pepys, M. B., Dash, A. C., Fletcher, T. C., Richardson, N., Munn, E. A., and Feinstein, A. 1978. *Nature (Lond.)* 273: 168-170.
13. Pepys, M. B. and Baltz, M. L. 1983. *Adv. Immunol.* 34: 141-211.
14. Riley, R. F. and Zontine, W. 1972. *J. Lab. Clin. Med.* 80: 698-703.
15. Stults, N. L., Lee, Y. C., Hoppe, C. A., Kawaguchi, K., Kohda, S., Takagahara, I., Koishi, T., and Liu, T. Y. 1987. *Anal. Biochem.* 161: 567-573.
16. Volanakis, J. E. and Kaplan, M. H. 1967. *Proc. Soc. Exp. Biol. Med.* 136: 612-614.
17. Yamamoto, S., Tagata, K., Nagahata, H., Ishikawa, Y., Morimatsu, M., and Naiki, M. 1991. *Vet. Immunopathol.* (in press)