PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 339-343 (1976)

Physical Relationship between Canine Factor VIII Coagulant Activity and Factor VIII-Related Antigen[1] (39541)

ROGER E. BENSON AND W. JEAN DODDS
(Introduced by C. A. Hall)

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

Canine factor VIII (FVIII), the plasma coagulant functionally defective in dogs with hemophilia A (1), can be isolated by gel chromatography in physiological buffer systems as a macromolecule with a molecular weight in excess of 10^6 daltons (2). The coagulant activity of canine FVIII (VIII-C) is recognized by its ability to correct the clotting defect of hemophilia A plasma (1). In addition, canine FVIII gives a precipitin reaction against a specific heterologous antibody, this precipitating determinant being designated FVIII-related antigen (VIII-RA) (3). A third property of canine FVIII is its ability to correct the defective ristocetin-induced platelet aggregation of plasma from dogs with von Willebrand's disease (4). Human, porcine, and bovine FVIII exhibit some or all of these same characteristics (5-7).

Because all three of these properties are found in the same chromatographic fractions after gel filtration (8, 9), it is possible that they represent three functions of a single molecule. However, recent studies have demonstrated that, as with human FVIII, canine VIII-C and VIII-RA can be segregated *in vitro* by high-salt-concentration chromatography (2). By preparing heterologous antisera against different components of the FVIII complex and coupling the antibodies to a solid-phase matrix, we have been able to study further whether FVIII is composed of more than one molecule. Differential removal of either VIII-C or VIII-RA from canine plasma by the insolubilized antibodies would suggest that they are distinct molecules. A preliminary summary of the data described here has been reported (10).

Methods. Canine factor VIII was prepared from the cryoprecipitate of 40 ml of plasma resuspended in 5 ml of 0.03 *M* barbital-buffered saline, pH 7.4, at a final concentration of 25 mg/ml. The crude FVIII preparation was chromatographed on a 2.5 × 43-cm 4% agarose column (Bio-Gel A-15m, Bio-Rad Laboratories, Richmond, Calif.), and eluted with the resuspension buffer at 4° and 20 ml/hr. The 5-ml fractions were monitored for VIII-C by the one-stage partial thromboplastin time method using canine FVIII-deficient substrate (11), and for VIII-RA as previously described (3). The two fractions that eluted at the end of the void volume and contained 70 μg of protein/ml and the maximum amount of VIII-C were pooled and stored in 1-ml aliquots at −40°.

A 4-kg crossbred Flemish giant-chinchilla rabbit was immunized with the gel-filtered canine FVIII preparation. The rabbit received eight weekly 0.5-ml intradermal injections of the FVIII preparation containing 2.5 to 5 units of FVIII (1 unit represents the VIII-C or VIII-RA level in 1 ml of a pool of equal volumes of plasma from eight randomly selected adult dogs, four of each sex) and 35 μg of protein mixed with an equal volume of sterile 0.23% $Al(OH)_3$. The animal was rested for 1 month, reimmunized once, and bled 10 days later. The serum obtained was first decomplemented by heating for 30 min at 56° and then absorbed with 10 mg Ca_3PO_4/ml at 25° for 20 min.

Canine hemophilia A plasma obtained from dogs in the Chapel Hill colony (1) was fractionated by the same cryoprecipitation and gel-filtration techniques. The hemophilia A plasma contained <0.01 U of VIII-C/ml and >1.0 U of VIII-RA/ml. The two chromatographic fractions that eluted at the end of the void volume and contained the maximum VIII-RA were pooled, aliquoted, and used to raise a rabbit antiserum as above.

[1] Supported by NIH Research Grant No. HL09902 awarded by the National Heart and Lung Institute, PHS/DHEW.

The anti-normal (AN) and anti-hemophilia A (AH) antisera were analyzed by immunoelectrophoresis in 0.9% agarose and rendered monospecific by absorption with fractions of normal canine plasma made low in VIII-C and VIII-RA by the method of Zimmerman *et al.* (12). The specificities of the absorbed antisera were compared by Ouchterlony immunodiffusion. An aliquot of the AN antiserum was absorbed once with an 8% ethanol concentrate of the hemophilia A plasma to produce a third type of antiserum (Hem-AN), which neutralized VIII-C but was devoid of precipitating antibodies. The ratio of absorbing hemophilic plasma to antiserum was 0.5 to 1.0 vol.

The avidities of the AN and AH antisera for VIII-RA were determined by comparing electroimmunodiffusion peak heights produced with constant antigen concentrations and varying antisera dilutions, as described by Kernoff and Rizza (13). The precipitating characteristics of these antisera were also compared by repeatedly using each to determine the VIII-RA levels of plasma from a normal dog and from dogs with hemophilia A and von Willebrand's disease.

The patterns generated by the antisera on crossed immunoelectrophoresis were used to characterize further their precipitating similarities. Each gel was prepared on a 95-mm^2 glass plate. One-half consisted of 0.9% Seakem agarose; the rest contained the same gel plus monospecific rabbit anti-canine FVIII. A 3-mm well was punched in the nonimmunogel 1 mm from the boundary between the two halves. Six to ten microliters of an 8% ethanol concentrate of plasma were loaded in the well and electrophoresed in the Gelman Deluxe electrophoresis chamber at 15 mA for 2.5 hr in a path parallel to the gel interface, then for an additional 3.0 hr into the immunogel at 25°.

The anti-VIII-C activities of the antisera were determined by a modification of the second stage of the inhibitor neutralization method of Denson (14). Equal volumes (0.25 ml) of pooled normal canine platelet-poor plasma (containing 1 U of VIII-C/ml) and dilutions of the antisera were incubated for 2 hr at 37°, then centrifuged for 15 min at 700*g*. The supernatant was assayed for residual AHF. The inhibitor unit is defined as the amount of inhibitor which will destroy 75% of the added factor VIII after a 2-hr incubation at 37°.

The globulin fractions of the monospecific antisera and nonimmune rabbit serum were isolated by half-saturation $(NH_4)_2SO_4$ precipitation, equilibrated in 0.05 *M* phosphate-buffered saline (pH 7.4), and concentrated against polyethylene glycol 20,000 to contain approximately 50 mg of protein/ml. Eighty milligrams of globulin from each antiserum and nonimmune rabbit serum were bound to 20 ml of packed Sepharose 2B beads activated with CNBr by the method of Cuatrecasas *et al.* (15). Unbound sites on the beads were blocked with 1.0 *M* glycine, followed by the standard acetate, urea, and $NaHCO_3$ washes. The antibody-coated beads were suspended to a 50% concentration in imidazole-buffered saline, pH 7.4.

The effect of the antibody and control beads on canine plasma was determined by mixing 0.60 ml of various combinations of nonimmune and antibody-coated beads with an equal volume of normal canine plasma in a 10 × 75-mm siliconized glass tube. After a 3-hr incubation at 37°, during which the tubes were inverted at 5-min intervals, the beads were separated from the plasma by centrifugation for 10 min at 700*g*. The supernatant was aspirated and assayed immediately for VIII-C and subsequently for VIII-RA by electroimmunoassay. Serial dilutions of the normal canine plasma, similarly incubated, were used as reference standards for both the coagulation and precipitation assays. The levels of VIII-C and VIII-RA after incubation with nonimmune beads were used as the control values in computing the percentage of residual VIII-C and VIII-RA.

Results. The absorbed AN and AH sera formed only one precipitin line when analyzed by immunoelectrophoresis, lines of complete identity by immunodiffusion (Fig. 1), and similar double-component precipitin patterns when analyzed by crossed immunoelectrophoresis. On electroimmunodiffusion 16% more AH antiserum was required to produce peak heights equal to those obtained with AN antiserum tested against the same normal canine plasma. Multiple VIII-RA determinations on three different canine plasma samples with both AN and AH

antisera gave values that were not statistically different ($P > 0.2$).

The absorbed AN antiserum contained 60 anti-VIII-C inhibitor units. No anti-VIII-C activity could be demonstrated by repeated neutralization assays using either the absorbed or nonabsorbed AH antiserum. When the AN antiserum was absorbed with hemophilia A plasma its precipitating avidity could not be detected by either Ouchterlony or Laurell methods, and its VIII-C neutralizing titer was decreased to 4 inhibitor units.

Thus, three types of anti-FVIII antisera were produced: (i) AN, which both neutralized VIII-C and precipitated VIII-RA; (ii) AH, which only precipitated VIII-RA; and (iii) Hem-AN, which only neutralized VIII-C.

The data from the three types of antibody–bead incubations are summarized in Table I. AN-globulin-coated beads at each of three dilutions removed similar amounts of VIII-C and VIII-RA from normal canine plasma. The same was true of AH-globulin-coated beads. In contrast, plasma incubated with 100% Hem-AN-coated beads retained 75.2% of VIII-RA but only 38.4% of the VIII-C. The VIII-C and VIII-RA levels of plasma incubated with the control beads were altered only to the levels predicted as the effects of dilution.

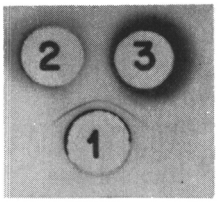

FIG. 1. Agar gel immunodiffusion. The wells contained: 1, normal canine plasma; 2, anti-normal canine FVIII; 3, anti-hemophilic canine FVIII.

Discussion. The precipitating antigen detected by the monospecific AN antiserum was considered to be analogous to human VIII-RA because of the antiserum detected: (i) no precipitin material in plasma from a patient homozygous for von Willebrand's disease and normal levels in healthy humans (H. R. Gralnick, personal communication); (ii) decreased levels of precipitin material in dogs from families with well-characterized von Willebrand's disease (3); (iii) elevated levels of precipitin material in dogs with hemophilia A (3); and (iv) precipitin material only in the void volume fractions of gel-filtered cryoprecipitates.

The precipitating material detected by the cross-reaction of AH antiserum with normal canine plasma appeared to be the same as that detected by AN antiserum on electroimmunoassay, double diffusion, and crossed immunoelectrophoresis. It was, therefore, also considered to be VIII-RA. The precipitating capacity of the AN antiserum was apparently abolished by its absorption with hemophilia A plasma, which left only the VIII-C neutralizing capacity.

Two of the three antibody systems appeared to remove VIII-RA and VIII-C simultaneously. Undiluted beads coated with the globulin fraction of an antiserum that precipitates VIII-RA but has no neutralizing capacity (AH-coated beads) removed about the same amount of VIII-C and VIII-RA from normal canine plasma, as did AN-coated beads, which have both anti-

TABLE I. VIII-RA AND VIII-C (%) IN NORMAL CANINE PLASMA AFTER INCUBATION WITH ANTI-FVIII-COATED SEPHAROSE BEADS.[a]

	Antibody coated to beads		
Antibody-coated beads (%)[b]	Anti-normal FVIII	Anti-hemophilic FVIII	Hemophilic-absorbed antinormal FVIII
100.0 FVIII-RA	28.1 ± 13.0	39.8 ± 12.1	75.2 ± 8.3
FVIII-C	32.7 ± 16.0	35.3 ± 16.1	38.4 ± 8.5
33.3 FVIII-RA	44.4 ± 17.3	69.0 ± 18.2	84.5 ± 11.7
FVIII-C	63.7 ± 23.2	82.2 ± 14.4	61.0 ± 15.4
16.5 FVIII-RA	67.0 ± 24.7	79.0 ± 9.6	106 ± 28.0
FVIII-C	86.3 ± 16.2	95.6 ± 5.1	74.2 ± 12.4

[a] Mean ± SD; $n = 6$. Each determination is the quotient of the final experimental value and control level determined with nonimmune globulin beads.

[b] Percentage of anti-FVIII-coated beads mixed with control globulin beads.

VIII-C and anti-VIII-RA bound antibodies. These results, especially those with AH-coated beads, suggest that VIII-RA and VIII-C may reside on a single molecule or a linked complex.

The removal of Hem-AN-coated beads of about twice as much VIII-C as VIII-RA is inconsistent with the above hypothesis. We feel justified, however, in placing less emphasis on these findings because production of the Hem-AN antibody required additional manipulation which might have influenced the results. It is also difficult to resolve how Hem-AN antiserum, which contains only 4 VIII-C inhibitor units/ml, can remove VIII-C as effectively as AN antiserum, which contains 60 inhibitor units/ml.

The concept that human VIII-C and VIII-RA are separate entities has received support from studies reported by Zimmerman and Edgington (16), who used a solid-phase antibody system, and by Hougie *et al.* (17), who used a double antibody technique. These results are in contrast to those reported in this study as well as to those of Hoyer (18) and Bird and Rizza (19), who have proposed that VIII-C and VIII-RA are part of the same molecule or complex because immunoprecipitates containing VIII-RA also contain VIII-C. Jaffé and Nachman (20) have suggested that all FVIII molecules contain VIII-RA, whereas a small percentage contain VIII-C.

Others have demonstrated that human VIII-C and VIII-RA can be physically separated by gel filtration (21), ion-exchange chromatography (22), and cryoprecipitation (23). In addition, it has been well documented that VIII-C is generated independently of VIII-RA after infusion of FVIII preparations into human patients and dogs with von Willebrand's disease (24–26; Bouma, Dodds, van Mourik, Sixma, and Webster, unpublished). These reports all demonstrate that under appropriate conditions, VIII-C and VIII-RA can be separated; however, there is still a lack of consensus about the physical relationship of circulating VIII-C and VIII-RA.

Our earlier studies (27) suggested that canine VIII-C and VIII-RA represent different antigenic sites on the canine FVIII complex but no speculation was made about their physical relationship. Most of the data presented in this study support the concept that *in vivo* VIII-C and VIII-RA may behave as a single macromolecule or complex.

Summary. Two of three insolubilized anti-canine FVIII systems removed similar amounts of VIII-C and VIII-RA from canine plasma. These data suggest that VIII-C and VIII-RA form part of one circulating complex.

The authors thank Dr. W. P. Webster, Chapel Hill, N.C., for providing the canine hemophilia A plasma and Joanne E. Kull for her assistance in collecting canine plasma samples and immunizing the rabbits.

1. Graham, J. B., Brinkhous, K. M., and Dodds, W. J., *in* "Handbook of Hemophilia, Part I" (K. M. Brinkhous and H. C. Hemker, eds.), p. 119. Excerpta Medica, Amsterdam (1975).
2. Owen, W. G., and Wagner, R. H., Thromb. Diath. Haemorrh. **27,** 502 (1972).
3. Benson, R. E., Bouma, B. N., and Dodds, W. J., Thromb. Res. **7,** 383 (1975).
4. Dodds, W. J., Blood **45,** 221 (1975).
5. Ratnoff, O. D., *in* "Progress in Hemostasis and Thrombosis" (T. H. Spaet, ed.), p. 39. Grune and Stratton, New York (1973).
6. Dodds, W. J., Webster, W. P., Brinkhous, K. M., Owen, C. A., and Bowie, E. J. W., *in* "Handbook of Hemophilia, Part I" (K. M. Brinkhous and H. C. Hemker, eds.), p. 141. Excerpta Medica, Amsterdam (1975).
7. Kirby, E. P., Martin, N., and Marder, V. J., Blood **43,** 629 (1974).
8. Weiss, H. J., Rogers, J., and Brand, H., Blood **41,** 809 (1973).
9. Gralnick, H. R., and Coller, B. S., Blood **46,** 417 (1975).
10. Benson, R. E., and Dodds, W. J., Amer. Soc. Hematol., Dallas, Tex., 160 (1975).
11. Dodds, W. J., J. Lab. Clin. Med. **76,** 713 (1970).
12. Zimmerman, T. S., Ratnoff, O. D., and Powell, A. E., J. Clin. Invest. **50,** 244 (1971).
13. Kernoff, P. B. A., and Rizza, C. R., Thromb. Diath. Haemorrh. **29,** 652 (1973).
14. Denson, K. W. E., Biggs, R., Haddon, M. E., Borrett, R., and Cobb, K. C., Brit. J. Haematol. **17,** 163 (1969).
15. Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B., Proc. Nat. Acad. Sci. USA **61,** 636 (1968).
16. Zimmerman, T. S., and Edgington, T. S., J. Exp. Med. **138,** 1015 (1973).
17. Hougie, C., Sargeant, J. E., Brown, J. E., and Baugh, R. F., Proc. Soc. Exp. Biol. Med. **147,** 58 (1974).
18. Hoyer, L., Nature New Biol. **245,** 49 (1973).
19. Bird, P., and Rizza, C. R., Brit. J. Haematol. **31,** 5 (1975).

20. Jaffé, E. A., and Nachman, R. L., J. Clin. Invest. **56,** 698 (1975).
21. Weiss, H. J., and Hoyer, L. W., Science **182,** 1149 (1973).
22. Baugh, R., Brown, J., Sargeant, R., and Hougie, C., Biochim. Biophys. Acta **371,** 360 (1974).
23. Hougie, C., and Sargeant, R., Lancet **1,** 616 (1973).
24. Bennett, B., Ratnoff, O. D., and Levin, J., J. Clin. Invest. **51,** 2597 (1972).
25. Muntz, R. H., Ekert, H., and Helliger, H., Thromb. Res. **5,** 111 (1974).
26. Weiss, H. J., and Sussman, J. J., Lancet **2,** 402 (1974).
27. Benson, R. E., and Dodds, W. J., Fed. Proc. **34,** 282 (1975).

Received March 22, 1976. P.S.E.B.M. 1976, Vol. 153.