

Immunologic Characterization of Canine Factor VIII

By Roger E. Benson and W. Jean Dodds

Canine factor VIII (FVIII) preparations isolated from cryoprecipitates by gel chromatography were pooled to provide one batch of antigen for simultaneous immunization of two rabbits and a goat. The goat and rabbit antisera had similar FVIII-neutralizing titers, but the latter had seven to ten times more precipitating titer for FVIII-related antigen (FVIII-RA). Absorption with material low in FVIII had little effect on the precipitating titer of the rabbit antibody, but it abolished the precipitating capacity of the goat antibody and caused a 20% reduction in the neutralizing titer of both antisera. Results obtained in the Laurell assay with the two different antisera were similar. This finding was true

whether the FVIII-RA levels were reduced, normal, or elevated, as well as for heat-treated and frozen-thawed plasmas. Both antisera were neutralized by the same canine plasma to a similar extent. Analysis of FVIII concentrates by crossed immunoelectrophoresis suggested that canine FVIII-RA was heterogeneous, with slow- and fast-migrating components. The presence of more than one antigenic site on the FVIII complex was also supported by the disparity between the FVIII-neutralizing and -precipitating titers of goat antiserum and by the demonstration that FVIII-RA, FVIII-neutralizing antigen, and procoagulant activity varied independently.

THE INTRICATE NATURE of the factor VIII (FVIII) complex is well recognized, as its hemostatic effects include both intrinsic coagulation and platelet retention in glass bead columns.¹ Hemophilia A patients express the coagulation defect, while individuals with von Willebrand's disease (VWD) usually have defects of both functions. That FVIII is a glycoprotein of apparently high molecular weight is not contested, but the precise subunit structure has yet to be established. Some researchers propose^{2,3} that FVIII has two noncovalently linked components of different size; others^{4,5} suggest that the FVIII complex consists of identical covalently bound subunits. In addition, some investigators^{6,7} believe FVIII to be a single multifunctional molecule, whereas others feel that preparations containing FVIII include several molecular entities.⁸⁻¹⁰

The availability of a closed colony of dogs with well-characterized hemophilia A and VWD¹¹ has permitted controlled genetic¹²⁻¹⁴ and infusion studies^{7,12} not readily performed with humans. This continuing resource has stimulated the present investigation of the structure of canine FVIII. The results provide immunologic evidence for separate antigenic and procoagulant sites in canine FVIII, as well as for the heterogeneity of the antigenic determinants.

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Submitted March 22, 1976; accepted May 28, 1976.

Supported by NIH Research Grant HL09902 from the National Heart and Lung Institute, PHS/DHEW.

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MATERIALS AND METHODS

Canine FVIII was prepared by gel filtration of plasma cryoprecipitates. Two hundred milliliters of frozen citrated platelet-poor dog plasma were allowed to thaw overnight at 4°C. One hundred and fifty milliliters of the supernatant were removed, and the remaining material was divided into four aliquots, which were centrifuged 5 min at 15,000 g and 0°C. Each pellet was resuspended in 5 ml of 0.03 M barbital-buffered saline,¹⁵ pH 7.4. Antihemophilic factor (AHF; procoagulant FVIII) activity was assayed by the one-stage partial thromboplastin time method using canine FVIII-deficient substrate as previously described.¹⁶ The total protein concentration of the FVIII concentrate was estimated by Goldberg refractometry (A. O. Instrument Co., Buffalo, N.Y.). The samples were stored at -40°C until needed.

Each aliquot was subsequently thawed and chromatographed on a 2.5 × 43 cm 4% agarose column (Biogel A 15M, BioRad Laboratories, Richmond, Calif.) with 0.03 M barbital-buffered saline, pH 7.4, at 4°C and 20 ml/hr. Ten-milliliter fractions were collected in polycarbonate tubes and assayed for protein by the method of Lowry¹⁷ and for AHF. The two fractions that eluted at the end of the void volume contained the maximum AHF and were pooled and stored in 1-ml aliquots at -40°C. An aliquot of the void volume fraction was also analyzed by sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis as previously described,¹⁸ as well as by agarose immunoelectrophoresis against rabbit anti-canine whole serum (Miles Laboratories, Elkhart, Ind.)

Two 4-kg crossbred Flemish giant-chinchilla rabbits and a 45-kg Alpine goat were immunized with the gel-filtered canine FVIII preparation. Each rabbit received eight weekly, 0.5-ml intradermal injections of the FVIII preparation containing 2.5-5 units of AHF activity (1 unit represented the AHF activity in 1 ml of a pool of equal volumes of plasma from eight randomly selected normal adult dogs, four of each sex) and 35 µg of protein mixed with an equal volume of sterile 0.23% Al(OH)₃. The goat received the same antigenic challenge according to the same regimen, except that the dosage was doubled.

Two weeks after the last immunization the animals were bled, and the serum was freed of complement by heating at 56°C for 30 min and then absorbed with 10 mg of Ca₃PO₄/ml at 25°C. The sera from the two rabbits were pooled, as both appeared to have similar anti-FVIII neutralizing and precipitating properties. Aliquots of the pooled serum were stored at -40°C.

The specificities of the goat and rabbit antisera were analyzed by Ouchterlony immunodiffusion at room temperature using 0.9% agarose (Seakem, Bausch and Lomb, Rochester, N.Y.) dissolved in high resolution buffer, 10 mg/ml, pH 8.7 (Gelman Instrument Co., Ann Arbor, Mich.). The 7-mm wells were refilled three times to keep them full for 8 hr, and then diffusion was continued for another 16 hr. The gels were washed in 0.05 M phosphate-buffered saline, pH 7.4, for 7 days, dried by warm air, and stained with Coomassie brilliant blue. The antisera, found to be polyspecific, were absorbed with an 8% ethanol precipitate of normal canine plasma rendered low in FVIII by a previous 3% ethanol fractionation by the method of Zimmerman.¹⁹ The ratio of rabbit antiserum to absorbing plasma was three volumes to one.

The apparent monospecificity of the absorbed rabbit antiserum was established by immunoelectrophoresis. Ten microliters of 3% ethanol-concentrated plasma were electrophoresed in 0.9% agarose on a 25 × 75-mm microscope slide at 30 V/slide for 2 hr at 25°C, followed by addition of antiserum and diffusion at 25°C for 16 hr.

The anti-AHF activities of the goat and rabbit antisera were determined by a modification of the second stage of the inhibitor-neutralization method of Denson.²⁰ Equal volumes (0.25 ml) of pooled normal canine platelet-poor plasma (containing 1 U/ml AHF) and dilutions of the antisera were incubated for 2 hr at 37°C and then centrifuged for 15 min at 3000 g. The supernatant was assayed for residual AHF. The inhibitor unit was defined as the amount of inhibitor which destroyed 75% of the added factor VIII after 2 hr of incubation at 37°C.

The precipitating titers of the two antisera were compared by a modification of the electroimmunoassay reported by Kernoff and Rizza.²¹ One-millimeter-thick gels of 0.9% agarose containing different antibody concentrations were poured next to each other on a 95-mm-square glass plate. Identical dilutions of normal dog plasma were placed in the 3-mm wells in each immunogel. The gels were electrophoresed at 25°C for 6 hr at 15 mA per gel with the Gelman buffer as the conducting medium. Since the peak height was inversely proportional to precipitating titer, the titers of the different antisera could be compared.

The FVIII-RA levels and antibody-neutralizing capacity of various canine plasmas were determined with both the goat and rabbit anti-FVIII antisera. The FVIII-RA of three normal plasmas (portions of each were assayed untreated, or heated to 56°C for 30 min, or frozen at -40°C and thawed three times), as well as three VWD plasmas and a hemophilic plasma, were quantitated as previously described.²² Once antiserum presumed to be measuring FVIII-RA became available, additional cryoprecipitates were gel-filtered as above, and the FVIII-RA levels of the fractions were determined.

The capacity of the VWD and hemophilic plasmas to block the AHF-neutralizing ability of the antisera was determined as follows: a 0.25-ml portion of either rabbit antiserum diluted 1:3 or goat antiserum diluted 1:5 was mixed with an equal volume of pooled standard or test plasma. The mixture was incubated for 90 min at 37°C, then for 30 min at 56°C; this was followed by a 15-min centrifugation at 4000 *g* at 25°C, the addition of the supernatant to 0.5 ml of normal dog plasma, and a final 90-min incubation at 37°C. Residual AHF was measured as described above. As a control, the effect of standard and test plasmas on nonimmune rabbit or goat serum was similarly evaluated.

Factor VIII concentrates from three normal, three VWD, and three hemophilia A dogs were analyzed by crossed immunoelectrophoresis. The concentrates were prepared from citrated plasma by precipitation at -3°C with 95% ethanol at a final concentration of 8%. The gel was prepared on a 95-mm square glass plate. One half consisted of 0.9% agarose; the rest contained the same gel plus 7.5% monospecific rabbit anticanine FVIII. A 3-mm well was punched in the gel without antibody 1 mm from the boundary between the two gels. Six to ten microliters of the FVIII-rich sample were loaded in the well and electrophoresed at 25°C in the Gelman 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 gel-containing antibody. Selected samples were analyzed by the same techniques, except that the antibody-containing gel was not poured until after the first electrophoretic step. In addition to plasma concentrates, normal canine plasma and plasma from a dog with pyometra (FVIII-RA level approximately 5.0 units/ml) were similarly analyzed at antibody concentrations from 1% to 10%. The gels were washed in phosphate-buffered saline for 48 hr, dried by warm air, and stained with Coomassie Brilliant Blue.

RESULTS

The canine FVIII concentrate prepared from cryoprecipitates contained 25 mg of protein and 5-10 units of AHF activity/ml. Filtration of 7.5 ml of the concentrate on a 4% agarose column resulted in a void volume fraction containing 0.3% of the protein with no significant loss of AHF activity. A single broad band of protein that eluted after the AHF peak contained the remainder of the filtered protein and formed gel-like clots when stored at 4°C. The pooled fractions containing the most AHF had 5-10 units of activity and 70 μ g of protein per milliliter, and were used to immunize the goat and rabbits.

Analysis of the void volume fraction by SDS polyacrylamide electrophoresis demonstrated a major protein band that did not enter the gel until reducing conditions (5 mM dithiothreitol) were employed. In addition, other faint bands were present.

The FVIII void-volume fraction did not generate any precipitin lines when examined by immunoelectrophoresis using rabbit anticanine whole serum. In a subsequent gel-filtered FVIII preparation tested with rabbit anti-canine FVIII, only the void-volume fractions contained precipitating antigen levels greater than those present in normal plasma. There was no detectable precipitating antigen present in the protein peak eluting after the AHF.

The complement-free and Ca₃PO₄-absorbed antisera were assayed for anti-AHF activity. At a 1 in 12 dilution, the rabbit and goat antisera both neutralized 75% of the added AHF when mixed with an equal volume of pooled

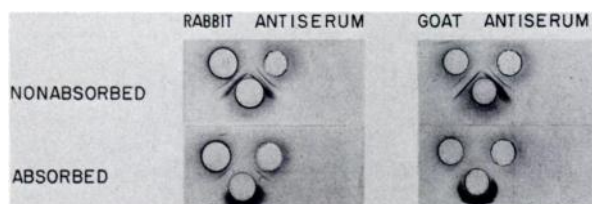


Fig. 1. Effect of absorbing rabbit and goat anti-canine FVIII antisera with an 8% ethanol precipitate of plasma low in FVIII activity. Center well in each panel contained the antiserum; upper left well, ethanol-concentrated normal canine plasma; upper right well, normal canine plasma. The contaminating precipitin antibodies of the goat antisera could not be removed without also eliminating the FVIII-RA reactivity. Both absorbed antisera had only slightly reduced AHF-neutralizing titers.

normal dog plasma. However, if the plasma-antiserum mixture was not centrifuged after the 2-hr incubation, the neutralization titer of the rabbit antiserum was fourfold less. In contrast, centrifugation only very slightly increased the inhibitor titer of the goat antiserum.

At least two strong and several weak precipitin lines were visible when the two antisera were diffused against whole canine plasma by Ouchterlony technique. The rabbit antiserum exhibited only one clearly defined line by both Ouchterlony diffusion and immunoelectrophoresis after absorption with a FVIII-poor ethanol precipitate of canine plasma (Figs. 1 and 2). However, we were never able to remove completely the contaminants from the goat antiserum without eliminating its precipitating capacity (Fig. 1). Analysis ($n = 5$) of the absorbing material indicated that the AHF and FVIII-RA contents were 0.115 ± 0.057 units and 0.276 ± 0.022 units.

Removal of the nonspecific precipitating antibodies from both antisera caused only a slight reduction in their anti-AHF activity. The neutralizing titers of both antisera dropped about 20% after absorption, and the precipitating antibodies in the goat antiserum were completely removed.

The precipitating titers of the antisera for FVIII-RA were compared by pouring three gels with different concentrations of rabbit anti-FVIII on a plate, along with a gel containing goat anti-FVIII, and comparing the peak heights

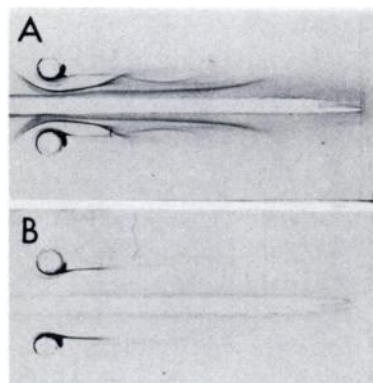


Fig. 2. Immunoelectrophoretic analysis of rabbit anti-canine FVIII. The wells contained ethanol-concentrated normal canine plasma. The trough contained (A) untreated antiserum; and (B) absorbed anti-FVIII.

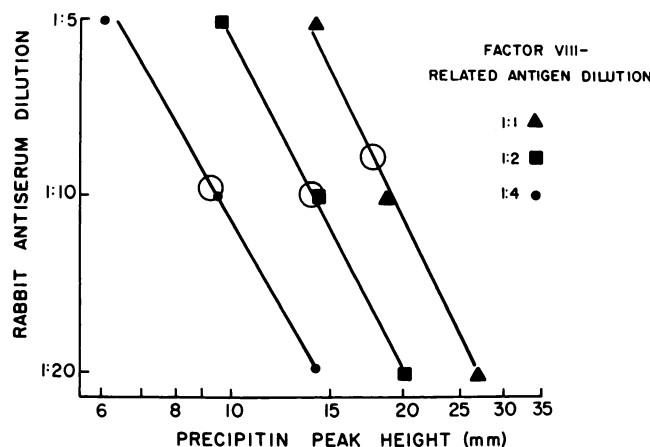


Fig. 3. Comparison of the precipitating avidity of goat and rabbit antisera for FVIII-RA. The large open circles represent the peak heights produced with undiluted goat antiserum, which were similar to those obtained with 1:10 diluted rabbit antiserum.

produced with constant antigen concentrations. The undiluted goat antiserum produced peak heights similar to those obtained for 1:10 diluted rabbit antiserum (Fig. 3).

Multiple FVIII-RA determinations on identical samples with both antisera gave similar values (Table 1). The peaks generated with the gels containing goat antiserum were clearly defined, making FVIII-RA quantitation possible even though the antiserum was polyspecific. Comparable results were obtained for the FVIII-RA of the three normal plasmas that were heat-treated or frozen-thawed. Although the FVIII-RA levels of these plasmas remained almost the same after processing, their AHF activities were greatly reduced (Table 2).

The FVIII-neutralizing antigen determinations with the goat antiserum resulted in standard curves that exhibited decreasing neutralization with increasing concentrations of pooled normal plasma. Both normal and VWD plasmas

Table 1. Per Cent FVIII-related Antigen and FVIII-blocking Antigen (Mean \pm SD of Six Determinations) With Two Different Antisera Raised Against the Same FVIII Immunogen

Antiserum	Pooled Normal Standard	Samples*			
		VWD ¹	VWD ²	VWD ³	Hem. A
Factor VIII-related antigen					
Rabbit	100	16 \pm 3	25 \pm 10	74 \pm 11	200 \pm 52
Goat	100	17 \pm 5	20 \pm 7	58 \pm 23	153 \pm 31
Factor VIII-blocking antigen					
Rabbit	100	Normal	Normal	Normal	Low
Goat	100	84 \pm 9	91 \pm 7	77 \pm 9	23 \pm 6

*VWD¹, VWD², and VWD³ are individual plasmas from dogs of the miniature schnauzer, golden retriever, and German shepherd strains, respectively.¹¹⁻¹³ Hem. A is a sample from a beagle hemophiliac. Factor VIII activities of these plasmas compared to the pooled normal standard were: VWD¹, 15%; VWD², 36%; VWD³, 48% and Hem. A, <1%.

Table 2. Per Cent FVIII-related Antigen and AHF Activity (Mean and Range) of Three Normal Canine Plasmas With Artificially Lowered AHF Activity

Assay	Treatment		
	None	Frozen-Thawed (3×)	Heated (56°C for 30 min)
FVIII-related antigen with			
Rabbit antiserum	100	95 (90-103)	91 (87-101)
Goat antiserum	100	91 (86-95)	82 (67-92)
AHF activity	100	51 (35-67)	<1*

*All were <1%.

neutralized the antiserum to a similar extent, while hemophilic plasma blocked the AHF-neutralizing capacity of the antiserum only slightly (Table 1). The neutralization of the rabbit antiserum by plasma samples was difficult to evaluate because rabbit serum has a demonstrable anti-AHF effect when mixed with canine plasma, which makes construction of a standard curve for blocking antigen difficult. However, the rabbit antiserum when mixed with either pooled normal, normal, or VWD plasmas gave significantly ($p < 0.01$) shorter clotting times (64.2 ± 1.7 sec; $n = 7$) in the partial thromboplastin assay than when mixed with hemophilic plasma (69.2 ± 2.7 sec; $n = 5$). The plasma samples gave identical residual AHF levels, whether mixed with nonimmune rabbit serum, nonimmune goat serum, or saline in the blocking antigen assay.

Analysis by crossed immunoelectrophoresis of the FVIII concentrates of canine VWD and hemophilia A plasmas did not reveal any major differences from FVIII concentrates of normal canine plasmas. The most commonly observed pattern (Fig. 4A) was a double-component precipitin formation with a relatively sharp peak at the origin connected to a second, faster-migrating arc. One ethanol-concentrated normal plasma concentrate consistently produced only the faster-migrating component (Fig. 4B). Pouring the antibody-containing gel before or after the first electrophoretic step did not change these results. Whole plasma samples produced indistinct patterns, except when plasma from an aged dog with pyometra (FVIII-RA 5.0 U/ml) was analyzed. This sample generated a precipitin formation similar to that of the plasma concentrates (Fig. 4C).

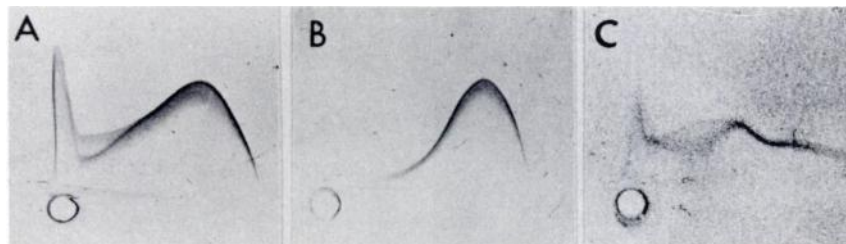


Fig. 4. Crossed immunoelectrophoretic analysis of canine FVIII concentrates. (A) The most commonly observed pattern among normal, VWD, and hemophilic samples. (B) This normal sample only contained one precipitin component. (C) The pattern generated by canine plasma with a FVIII-RA level of 5.0 U/ml.

DISCUSSION

The rabbit and goat antisera exhibited properties similar to heterologous antihuman FVIII.¹⁹ The absorbed rabbit antiserum was considered monospecific because it formed only one clearly defined precipitin arc by both Ouchterlony diffusion and immunoelectrophoresis and one continuous precipitin line when analyzed by crossed immunoelectrophoresis. The precipitating canine antigen detected by this antiserum was considered to be analogous to human FVIII-RA for several reasons. The antiserum detected: (1) no precipitin material in plasma from a patient homozygous for von Willebrand's disease, and normal levels in healthy humans,* (2) decreased levels of precipitin material in dogs from families with well-characterized von Willebrand's disease;²² (3) elevated levels of precipitin material in dogs with hemophilia A;²² and (4) precipitin material only in the void volume fractions of gel-filtered cryoprecipitate.

In addition to cross-reacting with FVIII-RA, both antisera neutralized AHF activity. However, the precipitating and neutralizing properties were not present in a constant proportion: the rabbit antiserum had ten times more precipitating avidity for FVIII-RA than the goat antiserum.

The lower precipitating titer of the goat antiserum was not unexpected, as we¹⁸ and others²³ have failed to produce goat anti-factor VIII of high precipitating avidity. The disparity between FVIII-neutralizing and -precipitating titers has been demonstrated previously for rabbit antiserum by Bouma et al.¹ and Shearn et al.,²⁴ who reported antisera to FVIII with relatively high precipitating and low neutralizing capacities.

The AHF-neutralizing titer of the rabbit antisera produced during this investigation also initially appeared to have a low neutralizing capacity, but the removal of insoluble complexes increased the neutralizing titer fourfold to equal that of the goat antiserum. The further reduction of residual procoagulant activity after removal of the immune precipitates suggests that the decrease of plasma AHF may involve more than one phenomenon.

The different reactions of these two species to the same antigenic challenge are not unexpected, although the generation of antisera with differential affinities for the FVIII complex suggests that canine FVIII is heterogeneous. There appear to be at least two sets of antibody specificities generated against canine FVIII, one which neutralizes AHF and a second which precipitates FVIII-RA. Additional support for this concept is provided by the finding that, following absorption, the AHF-neutralizing titer of the goat antiserum remains only slightly reduced despite elimination of the precipitating antibody.

While the rabbit and goat appeared to generate independent populations of antibodies against canine FVIII, both antisera gave comparable measurements for the FVIII-RA and FVIII-blocking antigen of the same test plasmas. These data suggest that both species produced populations of antibodies to canine FVIII, whose reactive sites responded in a similar manner.

Comparison of the AHF activity, FVIII-blocking antigen, and FVIII-RA levels of untreated, heat-treated, and freeze-thawed normal plasmas and of

*Gralnick H: Personal communication.

hemophilic and VWD plasmas demonstrated the complexity of canine FVIII. In no instance did these functions of FVIII appear to be increased or decreased in parallel, thus confirming their segregation.

The analysis of canine plasma concentrates by crossed immunoelectrophoresis with the absorbed rabbit antiserum supported the heterogeneity of the FVIII-precipitating antigen. The FVIII preparation examined had two immunologically related components: one of greater electrophoretic mobility and a second that migrated more slowly and generated a sharper peak. A single plasma sample with elevated FVIII-RA gave similar results. Evidence that the slower-migrating component was not an artifact of concentration was the repeated demonstration that one sample did not show this peak.

By crossed immunoelectrophoresis, hemophilia A and VWD concentrates generated patterns similar to those observed for the normal samples. Since no variant pattern was observed for the canine VWD samples analyzed, an electrophoretically aberrant molecule does not appear to be responsible for their hemorrhagic diathesis, as it is in some cases of human VWD.^{25,26}

In conjunction with our earlier studies,⁷ this investigation has confirmed the structural similarity of canine FVIII to its human counterpart. The canine FVIII complex is apparently of high molecular weight and readily generates heterologous precipitating and neutralizing antibodies. The antisera were used to demonstrate the independent nature of the FVIII-blocking and -precipitating antigens, as well as the segregation of the coagulation and FVIII-RA active sites. As in the case of human FVIII, it has not been clearly established whether the active and antigenic sites of canine FVIII are part of a multifunctional single molecule or are located on different molecules.

ACKNOWLEDGMENT

The authors wish to thank Joanne E. Kull for her assistance in collecting canine plasma samples and immunizing the rabbits and goat.

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