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FACTOR VIII-RELATED ANTIGEN IN CANINE HEMOPHILIA AND VON WILLEBRAND'S DISEASE: VARIATION WHEN MEASURED WITH DIFFERENT AGAROSES

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ABSTRACT

The source of agarose used in the Laurell electroimmunoassay was found to produce variations in the factor VIII-related antigen levels of plasmas from dogs with von Willebrand's disease and hemophilia A. Similar artifactual variations in antigen were not observed for normal canine plasmas.

INTRODUCTION

The level of factor VIII-related antigen (FVIII-RA) in human and canine plasma is normal or increased in hemophilia A and is usually reduced or absent in von Willebrand's disease (VWD)(1-3). This disparity is widely used to differentiate between these two diseases. However, variant forms of human VWD with normal levels of FVIII-RA have recently been reported and have been termed VWD type II (4,5).

The present investigation relates to an earlier collaborative study in which the levels of FVIII-RA in normal, VWD, and hemophilic dogs were determined independently in our two laboratories (2-3,6), using the Laurell electroimmunoassay (1). Initial results in our laboratory indicated that the plasma from 14 German shepherds with moderate VWD had an average of 48.1 ± 6.5% FVIII-RA and 41.5 ± 22.4% factor VIII activity, whereas plasma from seven different canine strains of severe hemophilia A had 127 ± 45% FVIII-RA and <1% factor VIII activity (2,3). Simultaneous studies in Doctor Bouma's laboratory using similar techniques and aliquots of the same normal pooled

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canine reference plasma, VWD plasmas, and antifactor VIII serum gave FVIII-RA levels 20-35% higher for the VWD samples (6). The difference could not be explained by technical or assay errors, as the coefficients of variation of the Laurell assay in the Utrecht and Albany laboratories were 10.5% (n = 20) and 16.0% (n = 10), respectively.

One of the variables in these two assays was a different commercial source of agarose. We therefore compared the heights of the precipitin peaks and the FVIII-RA levels obtained with three different agaroses. This report describes the results of these comparisons.

METHODS

The monospecific rabbit anticanine factor VIII serum used in these studies was prepared by B.N.B. (Utrecht antiserum). The antiserum was absorbed with 8% ethanol-precipitated canine plasma (1). On immunodiffusion the absorbed antiserum forms a single line of identity between normal canine plasma, canine factor VIII concentrate, and canine hemophilic plasma; a line of partial identity between normal human and normal canine plasmas; a faint line of identity with moderately affected canine VWD plasma; and no precipitin line with the plasma from a human patient with severe VWD (6). This antiserum also neutralizes factor VIII activity (3).

Another monospecific rabbit anticanine factor VIII prepared by R.E.B. (Albany antiserum) was used to repeat the initial determinations. Both the Utrecht and Albany antisera had similar properties, and the mean FVIII-RA levels obtained with them for three VWD plasmas and one hemophilic plasma were not statistically different at the 95% confidence level. In addition, similar levels of FVIII-RA were obtained with the absorbed and unabsorbed antisera.

Electrophoretic conditions and reagents other than the source of agarose were identical. Either Calbiochem[1], Seakem,[2] or Indubiose A-37[3] agarose (0.9%) was dissolved in Gelman High Resolution Buffer (Tris-barbital-Sodium-barbital, pH 8.8) diluted to 1.8 liters with distilled water. Ten ml of agarose with 0.3 or 1.2% of the absorbed Utrecht or Albany antisera, respectively, were cast on 80 x 95-mm glass plates and cooled overnight in a moist chamber at 4°C. Twelve 3-mm wells were punched in the agarose 25 mm from the end of the gel, and 6-µl samples were electrophoresed at 15 mA and 20 V/cm in a Gelman Delux Electrophoresis Chamber[4] for 6 hours at 25°C using the Gelman buffer and filter paper wicks as the conducting medium. The gels were washed in a 0.05 M phosphate-buffered saline, pH 7.4, circulating bath for at least 24 hours, dried by warm air and stained with Coomassie Brilliant Blue.

Plasma samples were prepared from venous blood drawn in plastic syringes containing one part 3.8% trisodium citrate to nine parts blood. The anticoagulated blood was placed in polycarbonate test tubes and centrifuged immediately at 20,000 g for 10 minutes at 4°C. The platelet-free plasma was stored at -40°C in 0.5- to 1.0-ml aliquots in either polycarbonate or silicone-coated test tubes. Samples were stored for a maximum of 8 weeks before use.

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[1]Calbiochem, San Diego, Calif.
[2]Bausch and Lomb, Rochester, N.Y.
[3]L'Industrie Biologique Francaise, Gennevilliers, France.
[4]Gelman Instrument Co., Ann Arbor, Mich.

The standard was a pool of equal volumes of plasma from eight random normal adult dogs, four of each sex, maintained in the same closed colony as the VWD and hemophilic dogs. This pooled canine standard was used throughout the study. The von Willebrand's and hemophilic dogs were from the closed colony previously described (2,7,8). One affected animal from each of three canine families with VWD (miniature schnauzer, golden retriever and German shepherd) and a beagle with severe hemophilia A were used. Aliquots of plasma from one bleeding of the VWD dogs were used for studies with the Utrecht antifactor VIII serum, whereas plasma from a second bleeding of the same animals was analyzed with the Albany antiserum. Sufficient aliquots of plasma from one bleeding of the hemophilic dog were available for the entire study.

Each Laurell plate contained the normal standard plasma in four dilutions the three VWD plasmas in two dilutions, and the hemophilic plasma in two dilutions. The diluent for all plasmas was the Gelman electrophoresis buffer. The final percent FVIII-RA was calculated by averaging the values for each sample dilution as compared to the curve obtained for the four dilutions of the normal standard. The same method was used to repeatedly determine antigen levels of two random normal dog plasmas. Two plates illustrating the comparable results obtained for FVIII-RA using unabsorbed and absorbed Albany antifactor VIII serum are shown in Fig. 1.

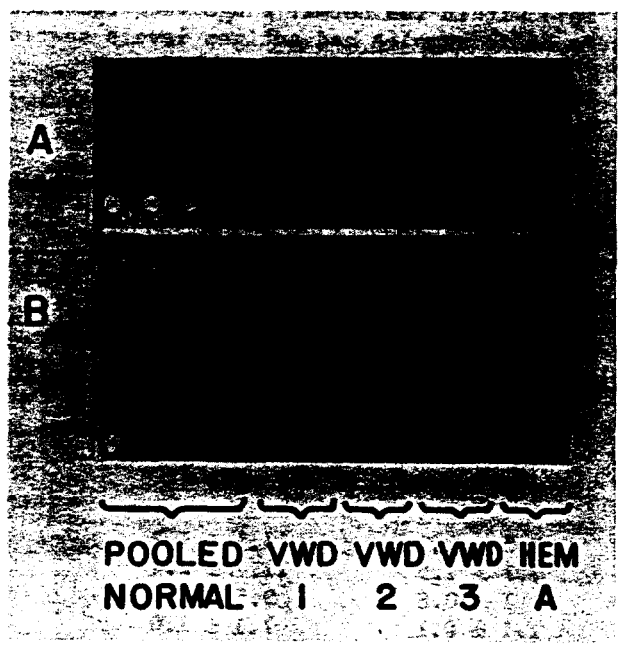

FIG. 1

Laurell electroimmunoassay with anticanine factor VIII - panel A with unabsorbed Albany antiserum, panel B with the same antiserum after absorption. The wells contained the following dilutions of plasma from left to right: A - pooled normal canine standard, 1:1, 1:2, 1:4, and 1:8, VWD-1 and VWD-2, 1:1 and 1:2; VWD-3 and Hem. A, 1:2 and 1:4. B - the same dilutions of the normal standard, VWD-1 and VWD-2, but 1:1 and 1:2 dilutions of VWD-3 and Hem. A. The FVIII-RA levels for the abnormal plasmas relative to the normal standard are 12, 23, 83, and 176% for panel A and 13, 16, 93, and 223% for panel B, respectively.

The factor VIII procoagulant activity of the samples was determined by one-stage partial thromboplastin assay using canine factor VIII-deficient substrate (2).

The sulphate content of the agaroses was determined by turbidometric technique after hydrolysis with hot 50% nitric acid (9).

RESULTS

Considerable variation was observed in the heights of the precipitin peaks produced with the three types of agarose using identical conditions and reagents. With both antisera and four dilutions of the pooled normal canine reference plasma, Seakem agarose consistently produced the highest peaks, Calbiochem the lowest (Table I).

The FVIII-RA levels of the three VWD dogs and one hemophilic dog obtained with the two antisera, as compared to that of the pooled normal canine standard, show consistent differences on different agaroses (Table II). By contrast, normal dog plasmas did not show this variation.

Indubiose agarose gave the highest FVIII-RA levels for the abnormal plasmas, 26 and 36% higher than the Seakem and Calbiochem agaroses, respectively. The FVIII-RA levels of the VWD samples electrophoresed on Indubiose agarose fell within the normal range with two exceptions (VWD-4, VWD-5), and the hemophilic samples had antigen levels nearly triple that

TABLE I

Peak Heights (mm) Obtained by Laurell Electroimmunoassay with Different Agaroses (Mean ± SD)

Dilution of pooled normal canine plasma*	Agarose		
	Calbiochem	Indubiose A37	Seakem
A. With Utrecht anticanine factor VIII			
	(n = 4)	(n = 6)	(n = 5)
1:1	7.5 ± 0.8	13.1 ± 1.2	20.0 ± 3.8
1:2	4.7 ± 1.5	11.7 ± 2.4	14.8 ± 3.6
1:4	2.1 ± 0.8	9.5 ± 2.2	10.6 ± 2.3
1:8	1.1 ± 0.1	7.1 ± 1.0	7.7 ± 1.4
B. With Albany anticanine factor VIII			
	(n = 6)	(n = 6)	(n = 6)
1:1	4.6 ± 1.4	18.6 ± 1.6	19.6 ± 2.7
1:2	2.4 ± 0.5	14.3 ± 1.1	15.1 ± 3.1
1:4	1.0 ± 0.3	9.4 ± 1.6	9.4 ± 1.2
1:8	0	6.2 ± 0.9	6.6 ± 0.9

of the normal standard. Some variation in FVIII-RA level was observed with different bleedings of the same animals (Table II).

The sulphate content of the Calbiochem agarose was found to be 0.50% (w/w), whereas that of the Seakem and Indubiose agaroses was <0.10% (w/w).

TABLE II

Factor VIII-related Antigen (%) with Different Agaroses (Mean ± SD)*

Sample	Agarose		
	Calbiochem	Seakem	Indubiose A37
A. With Utrecht anticanine factor VIII			
	(n = 2)	(n = 4)	(n = 3)
VWD-1	42 ± 13	53 ± 1**	69 ± 8**
VWD-2	87 ± 7	86 ± 12	113 ± 31
VWD-3	47 ± 3†	63 ± 11	77 ± 9†
Hem. A	168 ± 6	185 ± 75	298 ± 91
B. With Albany anticanine factor VIII			
	(n = 6)	(n = 6)	(n = 6)
VWD-4	0‡§	16 ± 3‡	18 ± 4§
VWD-5	0‡§	25 ± 10‡	31 ± 7§
VWD-6	70 ± 15	74 ± 11	85 ± 11
Hem. A	220 ± 37	206 ± 52	259 ± 46
Normal-1	90 ± 16	94 ± 2	94 ± 8
Normal-2	113 ± 10	95 ± 3	114 ± 13

*As determined by Laurell technique and compared to the pooled normal canine reference plasma, assigned a value of 100%. VWD-1, VWD-2, and VWD-3 are individual plasmas from dogs of the miniature schnauzer, golden retriever, and German shepherd strains of canine VWD, respectively (2,8). VWD-4, VWD-5, and VWD-6 are samples from different bleedings of these dogs. Hem. A is a sample from a beagle hemophiliac. Factor VIII activities of these plasmas compared to the normal canine standard were: VWD-1, 33%; VWD-2, 58%; VWD-3, 48%; Hem. A, <1%; VWD-4, 15%; VWD-5, 36%; and VWD-6, not measured.

The values indicated by the pairs of symbols show the following p: **, <0.01, †, <0.05; ‡ and §, <0.001.

DISCUSSION

The data indicate considerable variation in the height of the precipitin peaks obtained for normal canine plasma by Laurell electroimmunoassay with different types of commercial agarose (Table I). The peak height appears dependent upon the source of the agarose with the Seakem product, giving peaks more than double those obtained with Calbiochem agarose under identical conditions.

The differences observed in peak heights suggest that the rate of protein migration and formation of stable antigen-antibody complexes is influenced by the composition of the agarose used in the assay. It has recently been shown that protein migration in agarose is altered or increased by removal of sulphate groups (10). The sulphate content of Calbiochem agarose, which produced the lowest precipitin peaks in our study, was much higher than that of Seakem or Indubiose agaroses.

The agarose-associated variations in FVIII-RA levels (Table II) are greater than the error of the Laurell assay and cannot readily be explained, as conditions other than the antiserum were identical for all sets of plates. The FVIII-RA levels are not consistent with the heights of the peaks produced by the different agaroses: Seakem gave the highest peaks (Table I) and Indubiose the highest FVIII-RA (Table II). Furthermore, the variation was not observed for the two normal canine plasmas, and the FVIII-RA range for ten normal dogs was 68-163% for Calbiochem and 64-172% for Indubiose.

The increased antigen levels in samples from severely affected VWD dogs measured with Indubiose or Seakem agaroses did not overlap the normal range and thus would not preclude diagnosis of the otherwise well-characterized VWD of these animals (2,8). However, with these agaroses, samples from mildly or moderately affected dogs appear to have normal antigen levels and could therefore be classified as exhibiting the variant form of VWD described in man (4,5). Variations in the precipitating characteristics of different heterologous antihuman and anticanine factor VIII sera have been recently described (11,12), but it remains to be determined whether the findings reported here also apply to human VWD.

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