Further Studies of Canine von Willebrand's Disease

By W. Jean Dodds

Additional characterization of von Willebrand's disease (VWD) in a family of German shepherd dogs is presented. Genetic studies of three generations of affected dogs indicate that about 50% of the progeny are affected if one parent has VWD and about 60% if both parents have the defect. Some of these progeny manifested an incomplete form of VWD, suggesting autosomal dominant inheritance with variable expressivity. The disease become progressively less severe with advancing age and repeated pregnancies. Ristocetin-induced platelet aggregation was significantly reduced in VWD dogs as compared with normal, thrombopathic, and hemophilic carrier dogs. Immunodiffusion and electroimmunodiffusion studies with rabbit anticanine factor VIII showed the level of factor VIII-related antigen to be low in VWD dogs but present in increased amounts in hemophilic dogs. VWD affected dogs had markedly delayed hemostatic plug formation, but their plugs appeared normal by light and electron microscopy. Their platelet nucleotides, ATP/ADP ratio, and platelet protein content were normal. Platelet and fibrinogen survival times with [^{75}Se] selenomethionine were also normal, although platelets from VWD dogs incorporated more radioactivity than did those from normal dogs or from dogs with incomplete VWD.

IN 1970 WE DESCRIBED a family of German shepherd dogs with a hereditary bleeding disorder analogous to human von Willebrand's disease (VWD).[1,2] The defect was characterized by prolonged bleeding time, reduced factor VIII activity, abnormal platelet adhesiveness (retention), short prothrombin consumption time, overresponse of factor VIII levels after transfusion with normal or hemophilic plasma, autosomal inheritance with variable penetrance, and a mild to severe bleeding diathesis. The present report describes additional studies of the original family members and three successive generations of affected dogs.

FAMILY HISTORY

Our original family of VWD dogs consisted of seven mildly affected animals, five females and two males.[1] The propositus was severely affected, sterile, and died of an acute abdominal accident at 4 yr of age. Although the mildly affected dogs (28%-55% factor VIII) had not experienced bleeding episodes at the time of the original report, some of them subsequently developed large hematomas from minor trauma and required transfusion therapy.[3,4] The most frequently encountered hemorrhagic complications have been hematoma formation, intermittent lameness from periarticular bleeding, serosanguinous otitis externa, melena or protracted bloody diarrhea, prolonged estrual bleeding, and bloody postpartum vaginal discharges. The clinical expression of the disease appears to become progressively less severe with advancing age and repeated pregnancies.

Other moderately affected or normal adult dogs, closely related to the original family, have

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Table 1. Results of Genetic Studies in Canine VWD

Mating Types		No. of	Progeny (Live Births)										
			Males					Females					
Dam	Sire	Matings	N*	Mild or Moderate†	In-complete‡	Severe§	Total	N*	Mild or Moderate†	In-complete‡	Severe§	Total	Total
N	N	7	15	0	0	0	15	23	0	0	0	23	38
VWD	VWD	6	10	6	2	1	19	4	8	4	0	16	35
VWD	N	6	2	9	1	0	12	11	7	1	0	19	31
N	VWD	3	4	3	0	0	7	5	4	0	0	9	16
Total		22					53					67	120

*N, normal hemostatic tests (factor VIII activity, platelet retention, bleeding time).

†Mild or moderate, factor VIII 20%–60%, platelet retention <50%, bleeding time >12 min.

‡Incomplete (see text).

§Severe, factor VIII <20%, platelet retention < 10%, bleeding time > 30 min.

recently been studied or obtained for breeding purposes. Three additional generations of affected dogs have been raised from double-heterozygous and heterozygous-normal matings of the foundation and newly acquired stock. Three types of live progeny resulted from these matings: normal, mildly or moderately affected, and incompletely affected (Table 1). The one severely affected puppy bled to death at 10 days of age. The stillbirths were: two females from the normal-normal matings, ten males and seven females from the VWD-VWD matings, and one male and four females from the normal-VWD matings. The causes of death were unknown; no lesions were seen at autopsy.

The incomplete form of canine VWD is manifested in this family in two ways: by reduced factor VIII activity alone, or by low platelet retention with normal factor VIII levels and either a normal or prolonged bleeding time. All mildly or moderately affected dogs have a consistently prolonged bleeding time (> 10 min) regardless of their factor VIII level.

Several moderately affected dogs from this highly inbred family have recently developed rather bizarre secondary diseases. One bitch was born with VWD and a disease analogous to congenital lymphoedema (Milroy's disease) of man, cattle, and poodle dogs[5]; a 4-yr-old male with a history of chronic hypertrophic colitis and melena died of generalized miliary lymphomatosis; a 2-yr-old bitch was euthanized for acute renal failure secondary to Coombs' positive immune glomerulonephritis[6]; her brother died suddenly of intestinal volvulus; and several parasitefree affected dogs have demonstrated persistent eosinophilia, intermittent shifting lameness, and radiographic features indistinguishable from canine eosinophilic panosteitis.[7] The case reports of these animals will be presented elsewhere.

MATERIALS AND METHODS

Coagulation assays were performed on citrated plasma samples (1 part 3.8% trisodium citrate to 9 parts whole blood). Factor VIII was assayed by using canine factor VIII-deficient (hemophilic) plasma in a partial thromboplastin assay[1]; a pool of normal dog plasma from 20 random young adult dogs, ten of each sex, was assigned a value of 100% factor VIII. Bleeding times were determined on the subcutaneous tissue of the inner thigh with direct microscopic observation.[8] Hemostatic plug formation was studied by photomicrography, histology, and electron micrography.[8] The plugs were fixed in glutaraldehyde and postfixed in osmic acid for electron microscopy.

Platelet adhesiveness (retention) was measured by the Salzman and Bowie techniques,[9,10] modified as follows to facilitate reproducibility with canine blood: 6 ml of whole blood was drawn rapidly by clean venipuncture into a plastic syringe. A 1-ml aliquot was immediately dispensed to and mixed in a plastic tube containing 0.1 ml of 3.8% trisodium citrate. The remaining whole blood was passed by a syringe pump at 7 ml/min over a standard 1.0-g glass-bead column and collected in successive 1-ml aliquots in a series of plastic tubes, each containing 0.1 ml of the citrate anticoagulant. The platelet counts of the last three aliquots of blood from the column were averaged and subtracted from that of the precolumn sample to obtain the percentage platelet retention of the sample. The use of heparin anticoagulant for this assay was abandoned because canine platelets spontaneously aggregated in the presence of varying amounts of heparin (4–20 NIH U/ml), thereby preventing accurate platelet counts.

Ristocetin-induced platelet aggregation was measured in suspensions of washed rabbit platelets prepared by the albumin density-gradient technique of Walsh.[11] The assay technique of Weiss et al.[12] was modified for canine plasma as follows: Rabbit platelets were resuspended after the last albumin wash in modified Tyrode's solution (without divalent cations) and mixed with equal volumes of serially diluted normal and test canine platelet-poor plasmas. Ristocetin (Abbott Laboratories, Chicago, Ill.) was added to a final concentration of 2 mg/ml. The length of the lag phase and the degree of platelet aggregation were proportional to the final concentration of dog plasma in the system. Ristocetin-induced aggregation was compared in normal, VWD, and thrombopathic dogs[2,13] and in carriers of canine hemophilias A and B.[3,8]

This modified assay has the following advantages: a reproducible platelet suspension can readily be obtained from a strain of randomly bred rabbits; ristocetin-induced aggregation can be measured with plasma or plasma fractions from many species, including man; and lower concentrations of ristocetin will elicit aggregation with animal plasmas. By contrast, ristocetin aggregation of animal platelet-rich plasma requires concentrations of ristocetin (3–5 mg/ml) which also cause precipitation of fibrinogen and other plasma proteins.

Platelet protein content was determined on washed platelet suspensions by Miller's adaptation[14] of the techniques of Lowry et al.[15] Platelet nucleotides were measured on ethanol extracts by the method of Holmsen et al.[16]

Platelet and fibrinogen survivals were measured simultaneously with [^{75}Se] selenomethionine according to the method of Brodsky et al.[17] Normal and VWD dogs were given 4 μCi of ^{75}Se per gram of body weight. The platelet and fibrinogen survival times were each determined as the interval (in days) between the 50% points of peak radioactivity on the ascending and descending slopes of the curve.[17]

Agarose gel immunodiffusion and electroimmunodiffusion were performed with rabbit anti-canine factor VIII kindly provided by Dr. B. N. Bouma (Utrecht, Netherlands).[18] The antiserum was diffused against pooled normal, hemophilic, and VWD dog plasmas. Immunodiffusion utilized a template system with 2.5-mm wells, and electroimmunodiffusion was performed by the Laurell technique as described by Zimmerman et al.[19] Additional studies with these techniques will be reported elsewhere.

RESULTS

The primary bleeding time was prolonged in canine VWD (Table 2), and there was frequent rebleeding through the hemostatic plug, resulting in a prolonged secondary bleeding time. By contrast, the normal dogs stopped bleeding

Table 2. Bleeding Times by Direct Microscopic Observation in Normal Dogs and Dogs With Mild and Moderate VWD

Normal Dogs			VWD Dogs		
Dog	Sex	Bleeding Time*	Dog	Sex	Bleeding Time*
Donor	M	3'57"	Jorja†	F	14'30"
Igor	M	5'35"	Prince	M	22'10"
L-29†	F	2'37"	Wilhelm‡	M	17'13"
Lady¶	F	2'56"	Corey†‡	F	5'49"
Drew	M	2'00"	Sugar§	F	8'30"
Atlas	M	2'34"	Ulyssa¶	F	5'35"
			Phillip‡	M	13'00"
			Pretty Boy (G-3)	M	14'45"
			G-2†	F	13'30"

*Average from four to six vessels ranging from 50–150 μ in diameter.

†Virgin bitch.

‡Have incomplete form of VWD.

§Bitch after one pregnancy.

¶Bitch after three pregnancies.

Table 3. Ristocetin-induced Platelet Aggregation in Normal, VWD, and Thrombopathic Dogs and in Carriers of Canine Hemophilias A and B

Dog	No.	Final Plasma Dilution *			
		1:8	1:16	1:32	1:64
		Lag Time (sec)			
Normal	10	89 ± 15	132 ± 32	218 ± 32	312 ± 65
VWD	14	124 ± 30†	184 ± 71‡	307 ± 69†	455 ± 80†
VWD§	3	87 ± 11	119 ± 16	218 ± 23	279 ± 38
Hem. A	4	91 ± 23	146 ± 18	191 ± 31	275 ± 34
Hem. B	4	89 ± 12	128 ± 31	197 ± 31	277 ± 39
Thromb.	4	82 ± 32	103 ± 20	182 ± 80	274 ± 116
		Degree of Aggregation (%)			
Normal	10	100	75	25	25
VWD	14	65	50	25	25
VWD§	3	75	25	25	25
Hem. A	4	67	38	25	25
Hem. B	4	88	33	25	25
Thromb.	4	100	67	25	25

VWD dogs had 25%–60% factor VIII; proven hemophilic carriers had 42%–65% factors VIII or IX.
*Ristocetin, 2 mg/ml final concentration.
†$p = < 0.01$.
‡$p = < 0.05$.
§VWD bitches after two or more pregnancies.

within 2–5.6 min with little or no bleeding. The small skin incision healed readily in the normal dogs by first intention, whereas in several cases the incisions in VWD dogs became badly bruised or subject to hematoma formation, and healing was delayed. Although the formation of hemostatic plugs was markedly delayed in VWD dogs, histologic and electron micrographic sections of their plugs revealed no abnormalities. In some cases, the platelets in the plugs of VWD dogs contained large amounts of glycogen.

Ristocetin-induced platelet aggregation time was normal in thrombopathic dogs, carriers of canine hemophilias A and B, and three VWD bitches that had experienced two or more pregnancies (Table 3). By contrast, VWD dogs had defective ristocetin aggregation. Although the carriers of canine hemophilia A had factor VIII levels similar to those of dogs with moderately severe VWD, plasma from the VWD dogs was significantly slower to react in the ristocetin assay.

Table 4. Platelet Nucleotide and Platelet Protein Content of Random Normal Dogs, Breed-specific Normal Dogs, and Dogs With VWD

Platelet Nucleotides (μmoles/10^{11} platelets)	Normal Dogs		VWD Dogs
	Random	Breed-specific	
	(n = 10)	(n = 6)	(n = 21)
ATP	5.3 ± 1.1	5.2 ± 0.8	4.4 ± 1.3
ADP	1.9 ± 0.5	1.9 ± 0.6	1.6 ± 0.6
ATP/ADP ratio	2.9 ± 0.5	2.8 ± 0.6	3.1 ± 1.1
Platelet protein (mg/10^9 platelets)	2.2 ± 0.6 (n = 8)	—	1.9 ± 0.5 (n = 13)

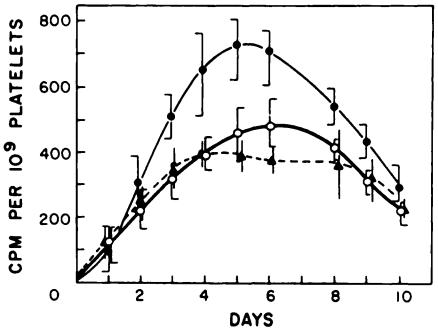

Fig. 1. Platelet survival with [^{75}Se] selenomethionine in six normal dogs (0–0), four dogs moderately affected with von Willebrand's disease (●—●), and two dogs with incomplete von Willebrand's disease (▲——▲). The data are shown as the mean ± SD survival curve for each group.

The platelet nucleotide and platelet protein content of our inbred German shepherds with VWD are compared with those of random normal dogs and normal related German shepherds in Table 4. The breed-specific control group was included to examine the possibility that a random population of normal dogs might have a different norm than that of an inbred line of purebred dogs. There were, however, no significant differences between the groups studied. The data indicate that, because of their lower ADP content, canine platelets have a higher ATP/ADP ratio than that reported in man.[16] These findings agree with those for other animal species.[20]

The platelet survival studies with [^{75}Se] selenomethionine in normal and VWD dogs are shown in Fig. 1 and Table 5. Although platelet survival time and protein content were similar in all three groups, there was significantly greater incorporation of radioactivity into the platelets of moderate VWD dogs ($p = < 0.01$). This difference may reflect a more rapid turnover of platelet proteins in dogs with VWD. The survival times obtained for ^{75}Se-labeled normal dog platelets are consistent with those reported previously[21] and with those in studies utilizing other radioactive labels such as chromium-51.[22] Plasma fibrinogen survival time was also determined during this study. The means for the six normal and six VWD dogs were 5.5 ± 0.9 and 6.3 ± 0.8 days, respectively. These results agree with those obtained for normal dogs with other labels such as sulfur-35[23] and iodine-131.[24]

When immunodiffused against rabbit anticanine factor VIII, plasma from dogs with moderate VWD produced a very faint precipitin line by 48–72 hr, whereas plasmas from normal and hemophilic dogs consistently developed a moderately heavy precipitin line within 36 hr. Electroimmunodiffusion studies using the Laurell technique showed that hemophilic plasmas from four different

Table 5. Platelet Survival of Normal and VWD Dogs

Dog	Survival Time, Mean (days)	Peak Radioactivity (cpm/10^9 platelets)*	Protein Content, Mean (mg/10^9 platelets)
Normal (n = 6)	7.4 ± 1.0	475 ± 40	2.6 ± 0.7
VWD, moderate (n = 4)	7.3 ± 0.7	710 ± 90	2.2 ± 0.8
VWD, incomplete (n = 2)	8.4 ± 0.5	378 ± 30	1.9 ± 0.1

*Peak occurred on days 5–7 for all groups.

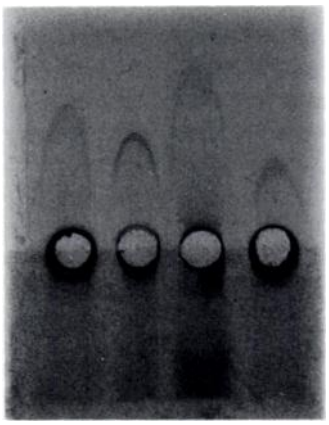

Fig. 2. A representative electroimmunodiffusion with rabbit anticanine factor VIII (0.5% of 1:10 diluted antiserum in 0.9% agarose). The antigen wells, reading from left to right, contained: undiluted pooled normal, 1:2 diluted pooled normal, hemophilic, and VWD canine plasmas. The hemophilic and VWD plasmas contained <1% and 33% factor VIII activity, respectively. Samples were electrophoresed for 24 hr at 4°C. The heights of the precipitin peaks measured from the centers of the wells were 11.0, 8.5, 13.0, and 6.5 mm, respectively.

canine families had increased amounts of factor VIII-related antigen as compared to pooled normal dog plasma, while 14 VWD plasmas had 41.5% ± 22.4% factor VIII activity and antigen levels of 48.1% ± 6.5% of the same normal pool (Fig. 2). These findings with respect to factor VIII-related antigen parallel those observed for normal humans and for humans with hemophilia and von Willebrand's disease.[18,19,25,26]

DISCUSSION

The additional genetic information available from the three new generations of affected dogs (Table 1) does not clarify the problem with regard to heterozygosity and homozygosity in VWD.[27] As originally reported,[1] the disease appears to be expressed in the heterozygous state because mildly and moderately affected dogs demonstrate the abnormalities of VWD in laboratory tests and also manifest a bleeding tendency.

Of the 35 live puppies born to affected parents, only one was severely affected; it bled to death at 10 days of age. By contrast, none of the 47 live progeny of one affected parent was severely affected. However, there were 17 stillbirths from the six double-heterozygous matings and five from the nine heterozygous-normal matings, whereas only two puppies were born dead in the seven litters from normal parents. Some of the dead progeny of the VWD dogs may have manifested a homozygous, lethal form of the disease.

Most of the surviving affected offspring manifested mild to moderately severe VWD, and the remainder had an incomplete form of the disease. Several of the moderately affected dogs experienced clinical bleeding episodes, some of which appear to have been spontaneous.

If one considers only the live progeny of VWD dogs (Table 1), about 60% of the offspring of double-heterozygous parents, and 50% of those born to heterozygous-normal parents had VWD or incomplete VWD. However, assuming that the stillborn puppies were affected, the incidence increases to 73% for the double-heterozygous and 57% for the heterozygous-normal matings. These data from a relatively small population approach the expected 3:1 and 1:1 ratios for the double-heterozygous and heterozygous-normal crosses of an autosomal dominant trait. We cannot conclude, however, that inheritance of canine VWD

is the function of a single gene locus. The phenotypic heterogeneity expressed in the affected progeny is more compatible with a polygenic inheritance, as has been suggested for humans and swine with VWD.[27-31]

The incomplete expression of human VWD has been recognized for many years.[27-30] More recent studies in man,[32] swine,[31] and dogs[1-3] indicate that the so-called recessive or carrier state of VWD can be determined fairly accurately by utilizing a combination of laboratory tests for hemostasis. Veltkamp and Van Tilburg[32] detected three heterozygous carriers of VWD among the asymptomatic relatives of a severely affected patient by statistically comparing their levels of factor VIII activity and factor VIII-related antigen. On the basis of their findings, these investigators suggested that factor VIII-related antigen is more directly related to the primary gene product of the von Willebrand locus than is factor VIII activity. The studies of Owen et al.,[31] using a combined hemostatic score obtained from the factor VIII level, platelet retention, and bleeding time of VWD pigs, showed that affected swine did not overlap the range for carriers but that about 40% of carrier pigs fell within the normal range. Our studies of canine VWD suggest that the disease can be manifested in three ways: severely affected (homozygotes, usually lethal), moderately to mildly affected (heterozygotes), and incompletely affected (heterozygotes). The latter two expressions of VWD may reflect the extremes of heterozygosity or variable penetrance of the abnormal gene.[27]

Von Willebrand's disease becomes progressively less severe with advancing age and during pregnancy.[28,30,33] In our experience, the factor VIII level of puppies 2-4 mo of age is about 80% of that in adult dogs. For this reason, the most accurate means for detecting the affected or partially affected dogs in a VWD litter is to use age-matched and/or littermate controls. The status of several affected dogs of both sexes over 6 yr of age has improved to the point where their hemostatic tests, except bleeding time, overlap the normal range. Affected bitches demonstrate a rapid increase in factor VIII activity and platelet retention during gestation. These parameters revert toward their base-line level after parturition but, in contrast to man,[33] never return to the baseline. After repeated pregnancies, several older VWD bitches have hemostatic tests that overlap the normal range (Tables 2 and 3). In these instances, we cannot separate the effects of age from those of multiple pregnancies. Perhaps the changes relative to age and pregnancy are more apparent in canine VWD because of the shortened gestation period and lifespan of the dog as compared with man.

The bleeding time abnormality in affected dogs (Table 2) parallels that observed in man and swine with VWD.[4,30,31,34] Primary and secondary bleeding times were both prolonged, and there was frequent rebleeding through the initial hemostatic plug. The frequency and duration of secondary bleeding were similar to those reported previously for canine hemophilias A and B, but in these diseases the primary bleeding time is normal.[8] In contrast to the paucity of fibrin in the hemostatic plugs of hemophilic dogs,[8] the plugs obtained from VWD dogs appeared normal by electron microscopy. Kahn et al.[35] reported quantitative and qualitative differences in the platelets of bleeder swine during viscous metamorphosis when compared with platelets from normal swine.

However, in our present studies and those of White,[36] human and canine VWD platelets appear to have normal ultrastructural features.

Dogs with VWD had abnormally delayed ristocetin-induced platelet aggregation (Table 3), as has been reported for man.[12,37,38] This defect was evident despite the relatively high factor VIII procoagulant activity of affected dogs. Weiss et al.[12] have similarly shown that the ability of plasma to support ristocetin aggregation is a function of its level of von Willebrand's factor and not necessarily its factor VIII procoagulant activity. The normal lag time for ristocetin aggregation of plasma from canine hemophilic carriers, in contrast to that from VWD dogs with similar factor VIII levels, supports this concept. Although the lag time was prolonged in VWD dogs as compared to hemophilic carriers, the degree of aggregation was reduced in both groups (Table 3). These findings may differ from similar studies in man which have dealt primarily with the degree rather than the rate of aggregation.[12,37,38]

Howard et al.[38] reported that they could separate human VWD patients into two groups by their ristocetin response: normal aggregation for patients with low normal platelet adhesiveness and mild to moderately reduced factor VIII activity, and reduced or no aggregation for more severely affected individuals. The moderate but significant increase in the lag time for ristocetin aggregation with VWD dog plasma may therefore reflect the relatively mild to moderate form of the canine disease. Some patients with Glanzmann's thrombasthenia also have reduced ristocetin aggregation,[37] but the response of four dogs from our family with thrombasthenic thrombopathia[2,13] was normal (Table 3).

Previous reports of several VWD patients[30,39,40] have described platelet nucleotide abnormalities and an increased ATP/ADP ratio, but the present investigation showed no abnormalities of total platelet protein, nucleotide content, or ATP/ADP ratio (Table 4). The VWD dogs also had normal platelet and fibrinogen survival times, although their platelets incorporated more radioactivity than normal dog platelets. This finding is not understood, but it suggests an increased platelet protein turnover, which may bear some relationship to the platelet function abnormalities of VWD.

The reduced level of factor VIII-related antigen in VWD dog plasma clearly establishes the analogy between this animal model and its human counterpart.[18,19,25,26,32] In addition, plasmas of hemophilic dogs were shown to contain increased amounts of factor VIII-related antigen. These data also parallel those described for man.[18,19,25,26]

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