

Von Willebrand syndrome induced by a *Bothrops* venom factor: Bioassay for venom coagglutinin

(Factor VIII complex/platelet aggregation/animal model)

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ABSTRACT Hereditary deficiency of the macromolecular Factor VIII complex results in classic von Willebrand disease in man and animals, a bleeder state characterized by loss of the multiple biologic activities associated with the Factor VIII complex, including the platelet-aggregating von Willebrand factor. The bleeding time is also long. Venom coagglutinin, a *Bothrops* factor that causes platelet aggregation *in vitro*, depletes the plasma of its von Willebrand factor. The rate of platelet aggregation is a function of the amount of the coagglutinin present. Based on this observation, a sensitive and quantitative assay for the venom coagglutinin was developed. We administered the purified *Bothrops* factor to normal pigs and dogs and induced a von Willebrand syndrome similar to the inherited disease. The plasma von Willebrand factor was severely depleted; the antihemophilic factor and the Factor VIII-related antigen were not depleted as much. The bleeding time was normal. During the induction phase of the syndrome, transient thrombocytopenia with a long bleeding time occurred. The pig was less sensitive than the dog to the effect of coagglutinin. The severity of the syndrome is determined by the amount of venom coagglutinin administered. It is suggested that the syndrome could be induced in any mammalian species because the plasma of all mammals tested *in vitro* is sensitive to the venom factor. This model provides another avenue for the study of the heterogeneity of the Factor VIII complex and the pathophysiology of its components.

The discovery of a *Bothrops* venom factor, venom coagglutinin (VCA), has provided a new probe for the study of plasma von Willebrand factor (vWF) (1). vWF is so named because it is absent or depleted in von Willebrand's disease (vWD), an autosomally inherited bleeding disorder. Of the multiple abnormalities in vWD, a lack of the platelet aggregating vWF and a prolonged bleeding time are most conspicuous. VCA appears to "activate" plasma vWF, following which platelet aggregation occurs. VCA has been uniquely valuable in providing a procedure for determination of plasma vWF levels in both man and animals and for measuring variations in this factor (1, 2). During the course of this platelet-aggregating reaction with VCA *in vitro*, the vWF was found to be depleted, leaving little or no residual activity in the plasma. This finding suggested that the administration of VCA *in vivo* could produce the pathophysiological counterpart of inherited vWD. However, a difficulty in testing this hypothesis was the lack of an assay for VCA. It had been observed that the rate of the vWF-dependent platelet aggregation was determined by the amount of VCA present (3). Further study of this relationship revealed that, within a certain range of macroscopic platelet aggregation times, the rate of the reaction was both a sensitive and a quantitative function of VCA concentration. A unit of VCA could be defined. The bioassay reported here appears to provide a reliable index for determin-

ing the potency of VCA preparations. With this procedure, we were able to study the dose-response relationship in animals given VCA.

In the animals studied, it was found that a von Willebrand-like disorder could be quickly produced with appropriate doses of VCA. The disorder was characterized by a depletion of plasma vWF and was similar in its characteristics to the phenotypic manifestations of one of the variants of vWD in man (4). Because of its acquired nature, the animal disorder is designated von Willebrand syndrome (vWS) rather than von Willebrand disease.

MATERIAL AND METHODS

Experimental Animals. Normal pigs and normal dogs of both sexes were used. The pigs, ages 2-3 mo, weighed 11.5-24 kg; the dogs, ages 4.5 mo to >5 yr, weighed 8.5-26.5 kg. The animals were from controlled breedings of stock maintained at the University's Francis Owen Blood Research Laboratory.

Preparation of VCA. VCA for the infusion experiments was prepared from the crude dried venom of *B. jararaca* (Sigma) by a chromatographic procedure (1) using a DEAE-cellulose column and elution with high-molarity NaCl. VCA fractions were made approximately isotonic (equivalent to 0.154 M NaCl) by dilution or dialysis and were further diluted with saline usually containing porcine or canine albumin (0.1%) (Sigma) for pig and dog experiments, respectively. The final preparations contained 75 units of VCA per ml and had no thrombin-like enzyme activity as judged by no clotting of a human fibrinogen (Kabi) solution in 120 min.

Infusion Studies. The general procedure for infusions has been described (5). VCA was injected intravenously into the cephalic vein of the pig and into the jugular vein of the dog at the rate of 1-4 ml/min. Blood samples for analysis were obtained from each animal once or twice on separate days prior to infusion, immediately before infusion, and at 20 min, 1, 2, 4, 8, 12, 24, 48, and 72 hr, and occasionally at 120 hr after infusion.

Assays for Factor VIII Complex Activities. vWF assays were performed by the VCA method with the macroscopic aggregation test procedure (3). For these studies, the test mixture contained equal parts of the following: 0.084 M imidazole-buffered saline, pH 7.35; citrated plasma, serially diluted with buffer; VCA; and dog platelets, formaldehyde-fixed and usually lyophilized, 800,000/mm³ (6). For pig plasmas, supplementary vWF determinations were made in some experiments with the macroscopic platelet aggregating factor (PAF) procedure (5) and with the macroscopic ristocetin test using lyophilized platelets (3). Plasma inhibitors of vWF were tested for by a modification

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Abbreviations: VCA, venom coagglutinin; vWF, von Willebrand factor; vWD, von Willebrand disease; vWS, von Willebrand syndrome; AHF, antihemophilic factor; PAF, platelet aggregating factor.

of the method of Brinkhous and Culp (7). Antihemophilic factor (AHF; Factor VIII:C) assays were performed by a modification of the partial thromboplastin time procedure (8) using canine hemophilic substrate; the unactivated test was used for pig plasma and the kaolin-activated procedure was used for dog plasma. Factor VIII-related antigen (VIII:R:Ag) in pig plasma was assayed as described (5). For the above three tests, citrated plasmas were stored at -70°C until tested. Reference citrated pig and dog plasmas, assigned a value of 1 unit/ml or 100% for each of the above variables, consisted of a plasma pool from three or four normal animals. Saline bleeding times were determined by the method of Mertz (9) at the same time intervals as indicated for blood sampling.

Platelet counts were determined on freshly collected venous blood by using the Unopette microcollection system (no. 5855, Becton Dickinson, Rutherford, NJ) according to the manufacturer's directions.

RESULTS

Depletion of Plasma vWF *in Vitro* with VCA. The plasma vWF activity was largely eliminated from pig plasma within a few minutes by the action of VCA in the presence of platelets (Table 1). Only traces of vWF remained in the supernatant plasma after removal of the aggregated platelets. This was also true for dog plasma. The VCA-mediated reaction appeared to be much more effective in depleting the plasma of vWF than were two other vWF-dependent reactions, aggregation with ristocetin and with PAF. When ristocetin (2 mg/ml) was substituted for VCA in the reaction mixture of test 1 (Table 1), residual vWF was 25%. In the PAF reaction, with lyophilized human platelets substituted for canine platelets and VCA omitted from the reaction mixture, residual vWF was 66%, a value in good agreement with a previous study with bovine plasma (10). Repeated stepwise platelet aggregation in both the ristocetin and the PAF reaction was required to decrease the vWF further, in contrast to the nearly complete depletion of vWF in the one-step procedure with VCA. The vWF in dog plasma is refractory to the ristocetin and PAF procedures (11).

Bioassay of VCA Activity. The bioassay of VCA is based on the observation that the rate of platelet aggregation under standard conditions with a constant amount of plasma vWF is dependent on the concentration of VCA (Fig. 1). On the arithmetic plot, the segment of the curve between 12 and 16 sec approaches linearity and was selected for measuring relative potency of VCA solutions. One unit of VCA is defined as that amount in 1 ml which causes a platelet aggregation time of 14 sec in a four-part standard test system, one part of which is VCA. From the data of Fig. 1 *Inset*, Table 2 was developed, which indicates the aggregation times resulting with varying VCA concentrations in the range 0.75–1.35 units/ml.

Table 1. *In vitro* depletion of vWF in pig plasma with VCA

Test	Test system components			Residual vWF, %
	Plasma	Platelets	VCA	
1	+	+	+	<5
2	+	+	0	100
3	+	0	+	100

vWF values are expressed as percentage of homologous reference plasma. In test 1, equal parts of plasma, buffer, fixed lyophilized canine platelets (1,600,000/mm³), and VCA (50 units/ml) were mixed. Buffer was substituted for the omitted reagent in tests 2 and 3. After incubation and agitation for 2 min, samples were centrifuged and residual vWF was immediately determined. vWF inhibitor assay was negative in each test.

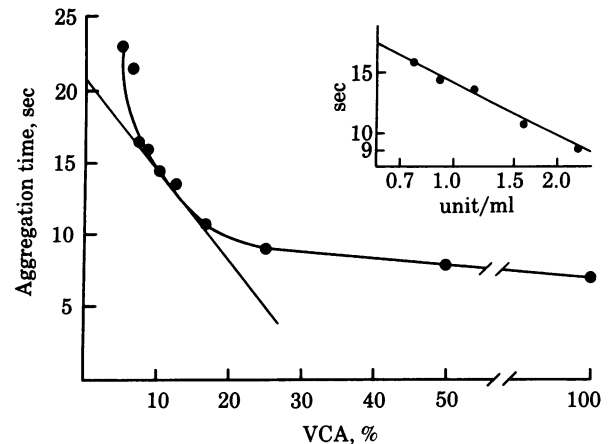


FIG. 1. Derivation of a VCA unit: Relationship of VCA concentration to platelet aggregation time. Test system consisted of equal parts of: (i) normal human plasma, reference pool stored at -70°C , designated as 1 unit of vWF per ml; (ii) buffer; (iii) lyophilized human platelets, a reference lot stored at -70°C and reconstituted, 800,000/mm³; and (iv) VCA solution. (*Inset*) Logarithmic replot of data in the range 9–16 sec, with conversion of VCA concentration to units/ml: 1 unit of VCA per/ml is equivalent to aggregation time = 14 sec.

An illustrative example of bioassay of an unknown preparation for VCA potency follows. The VCA solution was diluted such that at least two of the diluted samples caused aggregation in the range 12–16 sec (Table 2). The samples diluted 1:128 and 1:175 met this criterion with aggregation times of 13.0 and 15.5 sec, respectively (each an average of three replicate determinations). These time values are equivalent to VCA concentrations of 1.16 and 0.78 units/ml in the final test mixture. The VCA value based on the 1:128 sample would be

$$\begin{aligned}
 &128 \text{ (reciprocal of the initial dilution)} \\
 &\times 4 \text{ (reciprocal of 1:4 dilution in the test mixture)} \\
 &\times 1.16 \text{ (VCA in final test mixture from Table 2).} \\
 &= 594 \text{ VCA units/ml}
 \end{aligned}$$

For the 1:175 sample, the value would be 546 units/ml, for a mean of 570 units/ml.

Induced vWS. VCA in varying doses was administered to six normal pigs and six normal dogs. In the pig, in a typical experiment (Fig. 2A), the vWF activity decreased to <10% in the first 2 hr after injection and reached a minimum of <5% at 8 hr. Severe reduction of plasma vWF persisted for 24 hr, and then the vWF activity slowly returned to normal. At 48 hr, it was 50% of normal, and at 120 hr it was approximately 90%. When the vWF assays were also performed with the ristocetin procedure and human platelets, comparable reduced levels of plasma vWF were obtained at all time points.

Table 2. Derived conversion factors for bioassay of VCA

Platelet aggregation time, sec	VCA, units/ml
12.0	1.35
12.5	1.25
13.0	1.16
13.5	1.08
14.0	1.00
14.5	0.92
15.0	0.86
15.5	0.78
16.0	0.75

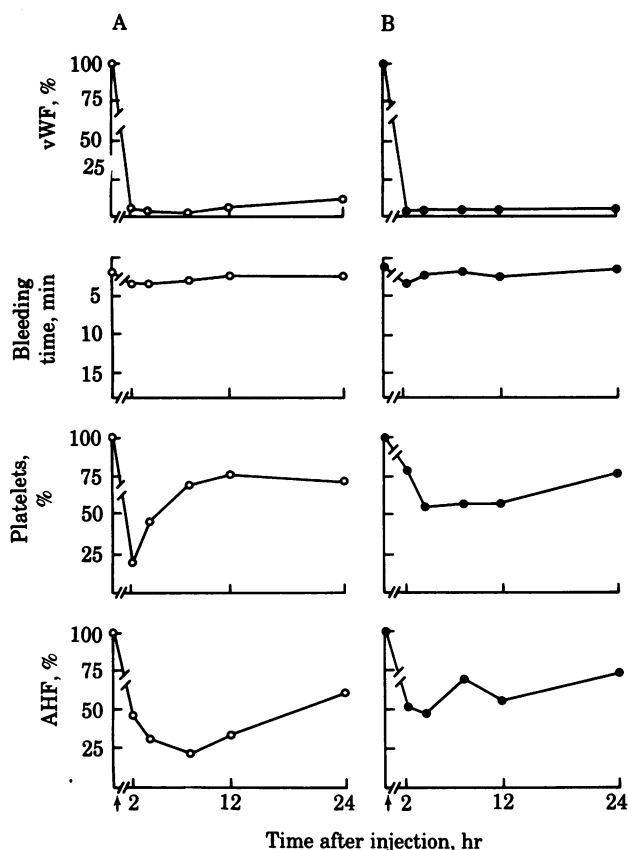


FIG. 2. Infusion of VCA into a normal pig (A) and a normal dog (B). The pig (male; 13.2 kg; 2 mo old) received 300 units of VCA per kg body weight. The dog (female; 26.4 kg; >5 yr old) received 86 units/kg. The preinfusion values for vWF, AHF, and platelet count were assigned a value of 100%, and the postinfusion values represent changes from these baselines. Arrows represent times of VCA administration.

In the normal dog (Fig. 2B), the response to the VCA infusion was similar to that in the pig. A lower dose was administered, 86 units/kg compared to 300 units/kg in the pig. The vWF levels were consistently $\leq 5\%$ of normal for nearly 24 hr and then returned to $>90\%$ at 72 hr postinfusion.

The bleeding time value at each postinfusion interval shown in Fig. 2 was normal or nearly so in both the pig and the dog. Even when the vWF activity was $\leq 5\%$ at 4–12 hr in the pig and at 2–24 hr in the dog, the bleeding time was not prolonged.

The platelet count after VCA infusion was depressed in both animals. Recovery of the platelet count to about 75% of preinfusion values was noted at 12 hr in the pig and at 24 hr in the dog.

AHF activity was also depressed in both species after injection of VCA. The AHF activity in the pig reached a nadir of 23% of the preinfusion value at 8 hr, when the vWF activity was $<5\%$. The AHF level had recovered to $>50\%$ at 24 hr. In the dog, the AHF level was lowest at 4 hr (36%) and had returned to $>50\%$ of the preinfusion value by 8 hr. Factor VIII-related antigen in the pig experiment was depressed moderately, as follows: 100%, preinfusion value; 85%, 2 hr; 73%, 4 hr; 56%, 8 hr; 46%, 12 hr; 50%, 24 hr; and 56%, 72 hr. Test for inhibitors of vWF in the 8-hr plasma sample from the pig was negative.

The dose–response relationship between the amount of VCA given and the maximal vWF depression after the injection is shown in Fig. 3. At equal VCA doses based on weight, there was a greater depression of vWF in the dog than in the pig. For example, at 150 units/kg, the vWF in the dog was decreased

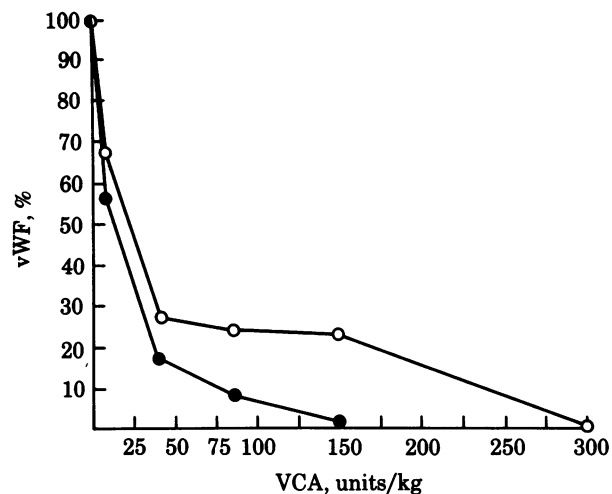


FIG. 3. Response of plasma vWF to infused VCA. Maximal depression of vWF after infusion in normal pigs (○) and normal dogs (●) is plotted against VCA. The data points at 43 units/kg for the pig and at 43, 86, and 150 units/kg for the dog each represent the average from two experiments; remaining points represent single experiments.

to $<5\%$ but in the pig it was decreased to only 25%. Maximal vWF reduction to $<5\%$ occurred in the pig only with a VCA dose of 300 units/kg.

The initial response to VCA injections in normal pigs and dogs was studied at 20 and 60 min. This time interval is designated as the induction period. Illustrative data are shown in Table 3. In pigs given VCA doses of 150 and 300 units/kg, there was an immediate and severe thrombocytopenia with a concomitant prolongation of the bleeding time to >15 min. With limited recovery of the platelet count at the 60-min interval, the bleeding time was only slightly prolonged. The bleeding time value had returned to within normal limits at the 2-hr interval (Fig. 2A). The thrombocytopenia was transient, being most marked at 20 min with continual return to normal thereafter. In contrast, vWF levels were just beginning to be depressed at the 20-min interval, after which they progressively declined to reach their lowest levels at 4–12 hr. In the dog, the greater sensitivity to vWF depression with VCA was also evident in the induction period. In both species, AHF during this time period was much less affected than was either vWF or platelets. The general well-being of the animals was maintained throughout the experiments, with no overt effects being observed. There were no hemorrhages.

DISCUSSION

An animal model for the vWS was induced in normal pigs and dogs by the intravenous infusion of the venom cofactor VCA which appears to cause activation and utilization of the plasma vWF. The finding that VCA effectively depleted normal plasma of its vWF activity in a one-step procedure (Table 1) led us to the testing of VCA *in vivo*. The syndrome is similar in many ways to inherited vWD in man and animals. In the fully developed induced syndrome, there was a severe deficiency of plasma vWF with accompanying modest reductions in coagulant Factor VIII (AHF) (Fig. 2) and Factor VIII-related antigen. The syndrome persisted for approximately 24 hr, after which the animals gradually returned to the preinjection state in 2–3 days. Because there were differences in the response of animals to various lots of VCA, the need for a procedure for determining relative potency of VCA preparations became important. Thus, a bioassay for VCA was developed.

Table 3. Induction period of vWS

Animal	VCA dose, units/kg	VWF, %		Platelet count, %		Bleeding time, min		AHF, %		
		20 min	60 min	20 min	60 min	20 min	60 min	20 min	60 min	
Pigs										
1	8	99	100	84	84	N	N	96	89	
2	43	64	33	73	99	N	N	91	70	
3	86	76	67	28	84	N	N	96	81	
4	86	73	43	11	34	N	N	119	83	
1*	150	77	57	<1	4	>15	6	98	92	
5	300	22	9	1	5	>15	7.5	40	43	
Dogs										
1	8	100	100	79	60	N	N	107	91	
2	43	98	32	5	10	8	9.5	98	91	
2*	43	67	40	4	17	12.5	N	108	66	
3	86	5	5	36	64	N	N	60	56	
4	150	≤5	≤5	4	27	>15	>15	88	87	
4*	150	≤5	≤5	5	20	13.5	>15	60	60	

The data shown as % are expressed in relation to preinfusion value = 100%. N, ≤5 min.

* Animals injected with VCA a second time within 4–9 days after first injection. Mean preinfusion platelet counts: pigs, 765,000/mm³; dogs, 430,000/mm³.

The VCA bioassay is based on earlier observations made during the development of the VCA test procedure for measuring plasma vWF (3)—namely, that the rate of platelet aggregation is a function of VCA concentration as well as of vWF concentration. Hence, it was necessary to maintain a constant amount of VCA for the performance of vWF assays. Conversely, in the bioassay of VCA, a constant amount of vWF was maintained in the test so that the aggregation times became a function of only VCA concentration (Fig. 1). The segment of the curve relating aggregation time to VCA concentration that approached linearity was used for the definition of a VCA unit and for the bioassay procedure. On the basis of these observations, a value of 1 unit of VCA per ml was arbitrarily assigned to that final concentration of VCA in the test mixture which caused an aggregation time of 14 sec. For a given VCA preparation, the unitage value appears to be reproducible within about ±10% on replicate determinations. This reproducibility is comparable to that observed with bioassays for most procoagulant factors, such as AHF. The macroscopic aggregation time method was chosen for the VCA assay rather than the aggregometry method (3) because the former appeared to detect smaller differences in VCA concentration. Table 2 provides factors for the determination of VCA units when there are minor deviations in the platelet aggregation time from the 14 sec or 1 unit VCA equivalent value. This bioassay method appears to be a quantitative and useful procedure for determining VCA concentration.

The pathophysiologic responses of the pig and the dog to infused VCA in the induction of vWS were similar (Fig. 2). At 2–4 hr there was a profound depression of the platelet aggregating vWF (<5%) which persisted for many hours. On the other hand, the bleeding time values were within or near normal values. This dissociation between plasma vWF levels and bleeding time values in the induced syndrome is in sharp contrast to their concordance in classic homozygous vWD in man (4), swine (5), and dogs (12), in which vWF is absent and bleeding time is indefinitely prolonged. There have been other reports of a dissociation of "bleeding-time factor" activity and vWF activity after transfusion in severe vWD in humans (13) and in swine (5). There are also reports of variant vWD with severely decreased vWF, moderately decreased AHF and Factor VIII-related antigen levels, and normal bleeding time (4), similar to the vWS-related antigen induced with VCA. The antigen level was depressed much less than in classic vWD, in which its value

typically corresponds to that of plasma vWF.

The most striking difference between pigs and dogs was in the dose–response relationships. To produce the fully developed syndrome with depleted vWF in the pig, VCA at 300 units/kg was needed, whereas in the dog, 86 units/kg, less than one-third of the pig dose, was sufficient. The dose–response curves (Fig. 3) demonstrate that the relative resistance of the pig to VCA compared to the dog was evident in all VCA dosages tested. Also illustrated in Fig. 3 is the fact that one can produce vWS of any degree of severity by proper regulation of the dose of VCA. The milder syndrome induced with lower doses of VCA persisted for shorter periods of time than did the severe syndrome with the higher doses.

The constellation of abnormalities observed during the induction period is different from that observed in the fully developed syndrome (Table 3). Thrombocytopenia occurred immediately, with partial recovery already underway at 1 hr. If the decrease in platelets was severe, the bleeding time was prolonged in both species of animals, but this association was not exact. One might have anticipated from the vWF depletion experiments *in vitro* (Table 1) that *in vivo* the plasma vWF values after VCA administration would immediately be decreased to their lowest levels, paralleling the decrease in platelets. This was not the case. Consistently, the vWF level continued to decline to its nadir in the 2–12 hr when the bleeding time anomaly had been corrected and the platelet count was approaching normal levels. This delayed depletion of vWF suggests that extravascular vWF stores may temporarily replete the plasma, only to be consumed by the continued action of the VCA.

Acquired vWS in humans has been reported many times (14). The pattern of Factor VIII activities is similar to that of severe inherited vWD, with low levels of plasma vWF, Factor VIII-related antigen, and AHF, with a long bleeding time. The syndrome usually develops as a complication of some other disease, such as systemic lupus erythematosus or hematopoietic malignancy. Many of the patients have a neutralizing antibody for vWF and, rarely, for AHF also, similar to the antibody vWF-inhibitor originally described in a multiply transfused homozygous vWD patient (15, 16). Thus, these patients with an acquired vWS appear to have an autoimmune disorder, very different from the VCA-induced vWS in pigs and dogs. The former usually appears to be due to the inhibitor effect of an antibody which neutralizes vWF, whereas the induced vWS appears to

be based on a massive consumption or utilization of vWF and, to a lesser extent, of AHF and Factor VIII-related antigen. In the stabilized state of the severe VCA-induced vWS, the bleeding time is normal or very slightly prolonged, whereas in the human acquired disease the bleeding time generally has been greatly prolonged. No vWF inhibitor was found in the induced severe syndrome as tested with a vWF activity neutralization test (7). Some humans with vWS likewise appeared not to have a neutralizing antibody (17). These patients could be analogous to the syndrome described here in animals, possibly a triggered vWF consumption induced by a released VCA-like factor during the course of disease. Should this be the case, then acquired vWS could be classified as either autoimmune usually with vWF neutralization or as a vWF-consumption syndrome due to release of a (physiologic) factor analogous in its action to VCA, comparable perhaps to diffuse intravascular coagulation in relation to the procoagulant biologic system.

The vWS syndrome was developed in two selected animal species because the plasmas of both were sensitive to VCA in the assay of vWF and were fully depleted of vWF in the presence of VCA and platelets. All plasmas of widely different mammalian species thus far tested (2) have likewise been sensitive to the effects of VCA. It would thus seem likely that the vWS could be produced in most mammalian species. Such animal models should be valuable for the further study of the pathophysiology and the nature of the functional and structural heterogeneities of the macromolecular Factor VIII complex.

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