

CONCISE REPORT

Use of Venom Coagglutinin and Lyophilized Platelets in Testing for Platelet-Aggregating von Willebrand Factor

By K. M. Brinkhous and M. S. Read

Previous procedures for measuring von Willebrand factor (vWF) in human plasma have usually utilized ristocetin and platelet-rich plasma or washed platelets. In this study, we describe a new approach for determination of vWF utilizing two new reagents. One is venom coagglutinin, a recently recognized component of *Bothrops* and other venoms, which aggregates platelets in the presence of vWF. The other is lyophilized fixed human platelets. The rate of platelet aggregation was found to be a function of both plasma vWF and venom coagglutinin concentration. By keeping the venom factor constant, simple and reliable test procedures for vWF were devised. Both a macroscopic test procedure and aggregometry were used for measuring platelet aggregation, with similar findings. Results obtained with the venom coagglutinin reagent were compared with those obtained with ristocetin. With the venom factor, normal plasmas from 20 subjects had a range of 70%–133% vWF and a mean of 96% of a reference plasma, while with ristocetin, the values were 63%–131%, mean 101%. Plasmas from seven von Willebrand disease patients gave comparable reduced values for vWF with venom factor and with ristocetin. The venom coagglutinin and dried platelet reagents appear to provide the basis for simple and reliable procedures for determination of plasma vWF.

A NEW platelet-aggregating activity in the venom of several species of snakes, particularly in many members of the *Bothrops* species, has recently been described.¹ This venom activity has been designated venom coagglutinin and requires the presence of the same plasma factor that promotes platelet aggregation or agglutination with ristocetin for its action. This plasma factor is known by various terms, including ristocetin cofactor or platelet-aggregating von Willebrand factor (vWF), since it is deficient in subjects with severe von Willebrand disease (vWD). Like ristocetin, the venom coagglutinin can be used in qualitative or quantitative tests for vWF.¹ Also like ristocetin, the venom coagglutinin causes aggregation of formaldehyde-fixed platelets, freshly prepared isolated platelets, and platelets in platelet-rich plasma. Unlike ristocetin, the venom coagglutinin does not cause plasma protein precipitation, which can be bothersome in assaying for plasma vWF.^{2,3} Recently, it was demonstrated that the ristocetin test for vWF can be considerably simplified if lyophilized fixed platelets are used instead of other platelet preparations.⁴

In this study we report on the use of venom coagglutinin with lyophilized platelets for screening tests and bioassay for vWF and present a comparison of results obtained with the venom factor and with ristocetin.

MATERIALS AND METHODS

Buffer. Imidazole-buffered saline was used as a diluent in all studies and consisted of 0.084 M imidazole (Sigma, St. Louis, Mo.) and 0.154 M NaCl, pH 7.35.

Citrated plasmas. Citrated plasmas were obtained by centrifuging citrated whole blood (8 ml mixed with 1 ml 3.2% trisodium citrate) from normal subjects and from patients with vWD. Plasmas were stored in 1 ml aliquots at -20°C before testing.⁵ Normal human reference plasma consisted of a mixture of equal parts of citrated plasma obtained from 10 normal subjects, aged 20–30 yr, 5 males and 5 females.

Venom coagglutinin. Venom coagglutinin was prepared from dried venom of *B. jararaca* (Sigma, St. Louis, Mo.) by chromatography on a DEAE-cellulose column as described previously,¹ lyophilized, and stored at -20°C until used. Reconstitution and further dilutions were made with buffered saline. The preparation was free of fibrinogen-clotting activity. For screening and bioassay procedures, the reconstituted venom fraction was diluted to give an aggregation time of 7–9 sec in the macroscopic screening test (see below).

Ristocetin. Ristocetin (Abbott Laboratories, Chicago, Ill.) was dissolved in buffered saline to give a stock solution of 2.4 mg/ml for macroscopic tests and of 30.6 mg/ml for aggregometry.

Lyophilized human platelets. Fixed human platelets were prepared by a modification of the method of Allain et al.⁶ and then lyophilized as previously described.⁴ A platelet suspension was made by adding imidazole buffer to the dry platelet mass to give a platelet concentration of 800,000/ μl .

Screening tests and bioassay procedure for platelet-aggregating vWF. The screening tests for vWF were performed with either venom coagglutinin or with ristocetin in a macroscopic aggregation test or in a Payton aggregometer, 37°C , with a recorder (Recordall series 500, Fisher, Pittsburgh, Pa.) set at 0.01 V. For the macroscopic test,⁵ a four-part system consisted of 0.025 ml each of plasma, imidazole buffer, resuspended lyophilized platelets, and venom coagglutinin or ristocetin. Aggregation times were determined by the tap tube method, in which the tube (10.75 mm), containing the four reagents, is tapped rapidly with a finger. The time of the first appearance of macroscopic platelet aggregates in the film of fluid flowing down the wall of the tube is recorded. For the aggregometer screening test, a mixture consisting of 0.3 ml buffer, 0.1 ml platelet suspension, 0.1 ml plasma, and 0.01 ml venom coagglutinin solution or ristocetin was employed.

For the bioassay of vWF, the four-part test mixture described

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above for the macroscopic screening test was used.⁵ Plasma was serially diluted and the vWF concentration was estimated in terms of a normal reference plasma as described previously.

RESULTS

The data selected for presentation illustrate the use of the two recently described reagents, venom coagglutinin and lyophilized platelets, for study of vWF. In the first set of studies, the effect of plasma concentration and venom coagglutinin concentration on the rate of platelet aggregation was studied with either the macroscopic aggregation test or the aggregometer. Figure 1 depicts one experiment in which serial dilutions of normal plasma (twofold dilutions) and of venom coagglutinin (tenfold dilutions) were used in the macroscopic aggregation test. Also tested in addition to normal plasma were homozygous vWD plasma and a mixture of equal parts of normal plasma from a vWD patient with an acquired antibody inhibitor of vWF.⁷ Plasmas containing vWF caused aggregation of platelets with venom coagglutinin, but in its absence no aggregation occurred, even if the observation periods were extended to 5–10 min. The time of platelet aggregation was progressively prolonged as the plasma was diluted, i.e., the aggregation time varied inversely with the amount of vWF. If the amount of plasma was kept constant, the rate of platelet aggregation was likewise progressively prolonged as the amount of venom factor was decreased. Plotting the data on a semilog scale (Fig. 1), the relation between aggregation time and venom factor concentration was linear within limits. In other experiments, platelet aggregation still occurred with the coagglutinin venom frac-

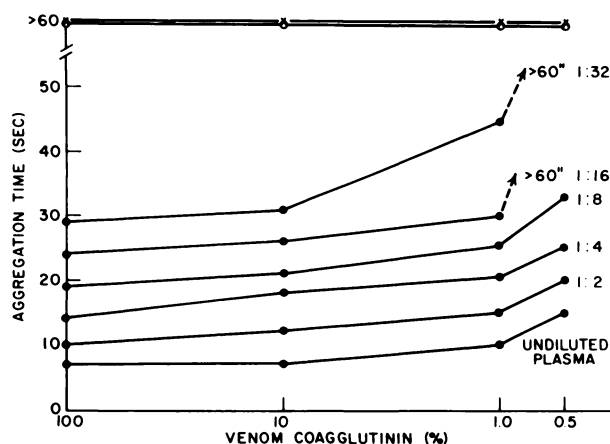


Fig. 1. Effect of varying concentrations of plasma and of venom coagglutinin on the rate of platelet aggregation observed macroscopically. Normal plasma serially diluted as indicated (●—●), severe vWD plasma (x—x), and a mixture of equal parts of normal plasma and a severe vWD-inhibitor plasma (o—o). Test mixture consisted of an equal part (0.025 ml) of buffer, plasma, coagglutinin, and platelets.

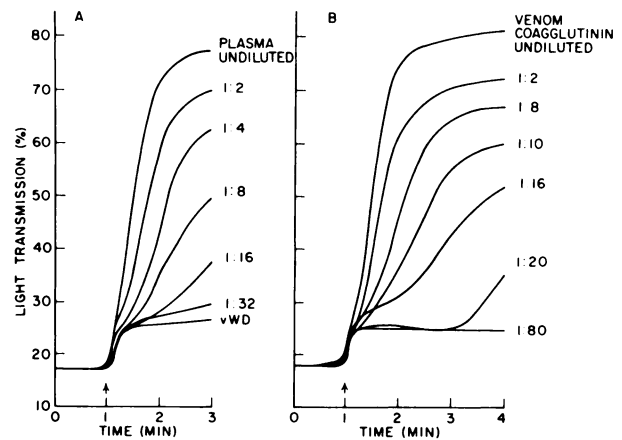


Fig. 2. Effect of varying concentrations of plasma and of venom coagglutinin on the rate of platelet aggregation observed with the aggregometer screening test (see Materials and Methods). Direct overlay tracings of aggregometer graphs shown. (A) Effect of normal reference plasma serially diluted and of severe vWD plasma (lowermost tracing), with constant amount of venom fraction. (B) Effect of varying amounts of venom coagglutinin and a constant amount of undiluted reference plasma. Arrows indicate time of addition of venom coagglutinin.

tion diluted up to 1:2000, but the aggregation times were in the range of 45–210 sec.

The effect of these two variables, concentrations of venom coagglutinin and of plasma, were studied in similar experiments with the aggregometer. Tracings are shown in Fig. 2A, with the amount of venom coagglutinin kept constant and the normal reference plasma serially diluted, and in Fig. 2B, where the venom coagglutinin concentration varied while the amount of plasma was kept constant. Light transmission increases with increased concentration of plasma vWF (Fig. 2A) or with increased concentration of coagglutinin (Fig. 2B) over a wide range.

In a second set of studies, the use of venom coagglutinin with lyophilized platelets was compared with ristocetin and lyophilized platelets to determine the level of plasma vWF. Table 1 illustrates the use of these sets of reagents in the macroscopic screening test for impaired vWF-dependent platelet aggregation. With venom coagglutinin, plasma of the mild vWD

Table 1. Macroscopic Screening Test for vWF: Venom Coagglutinin Compared to Ristocetin

Plasma	Platelet Aggregating Agent	Platelet Aggregating Agent	
		Venom Coagglutinin (sec)	Ristocetin (sec)
Type	Concentration		
Normal	Undiluted	7.5	8.0
vWD, mild	Undiluted	15.8	14.8
vWD, severe	Undiluted	>60.0	>60.0
Normal	1:4	15.4	13.6
vWD, mild	1:4	25.8	21.0
vWD, severe	1:4	>60.0	>60.0

patient gave a macroscopic aggregation time considerably longer than the normal control, regardless of whether undiluted or diluted (1:4) plasma was used. Similar results were obtained with ristocetin. Bioassays of vWF of this patient with either venom factor or ristocetin gave values of 26%. With plasma from a severe vWD subject, no aggregation was observed with either set of reagents. Not illustrated are the results obtained with the aggregometer screening test, but the tracings were similar to those obtained with undiluted plasma, 1:4 plasma, and vWD plasma (Fig. 2A). Also, similar curves with plasmas were obtained if ristocetin was used instead of the coagglutinin.

Bioassays of plasma for vWF were also performed with the macroscopic procedure using lyophilized platelets and either venom coagglutinin or ristocetin (Table 2). The plasmas were obtained from a group of normal subjects and of von Willebrand disease patients. For the normal subjects, it will be noted that there is a rather close correspondence in value with the two reagents, venom coagglutinin and ristocetin. The

Table 2. Bioassay of vWF in Normal and von Willebrand Disease Plasmas: Venom Coagglutinin Versus Ristocetin

Plasma		Aggregating Agent	
		Venom Coagglutinin (% vWF)	Ristocetin (% vWF)
Subject	Sex		
Normal subjects			
Reference		100	100
C.F.	M	124	131
L.M.	M	73	75
D.B.	M	100	100
R.W.	M	100	115
S.T.	M	77	100
A.B.	M	100	100
R.B.	M	100	100
B.B.	M	124	122
R.W.	M	94	101
C.H.	M	78	63
J.S.	F	100	100
K.K.	F	73	78
N.C.	F	133	122
J.P.	F	83	89
C.S.	F	100	115
V.T.	F	100	117
B.W.	F	79	100
M.L.	F	70	69
J.S.	F	78	100
N.C.	F	124	122
Mean \pm SD		95.5 \pm 19.2	100.9 \pm 18.8
von Willebrand disease			
L.C.	F	36	53
M.N.	F	21	24
K.K.	M	50	56
M.C.	M	8	5
V.K.	F	<5	<5
B.B.	F	<5	<5
F.M.	M	<5	<5

mean values for the normal subjects obtained with ristocetin were slightly higher than those obtained with the venom factor, but the standard deviation was much the same. No sex difference in vWF levels was noted in this small group of subjects. The range of values with venom coagglutinin was 70%–133% and with ristocetin 63%–131%. For seven vWD patients, the vWF values ranged from <5%–50% with venom coagglutinin and from <5%–56% with ristocetin.

DISCUSSION

The venom coagglutinin¹ offers a test reagent other than ristocetin for the study of von Willebrand factor-dependent platelet aggregation and the reactivity of plasma vWF. In these studies, it was found that the rate of platelet aggregation was determined not only by the concentration of vWF in plasma but also by the amount of venom coagglutinin in the test mixture, with the final platelet concentration being kept constant (200,000/ μ l). For the rate of aggregation to reflect the vWF concentration, the amount of venom coagglutinin must be kept constant (Fig. 2). For regular testing of vWF, an amount of coagglutinin was selected that caused platelet aggregation with undiluted human plasma in 7–9 sec. While aggregation occurred with as little as 8000-fold final dilution of the venom fraction, the aggregation time was prolonged and the platelet aggregates were small in size. Both the macroscopic test with direct visualization of platelet aggregation and the aggregometry procedure with determination of increased light transmission as aggregation proceeds were suitable for study of plasma vWF with the new venom factor.

The use of lyophilized fixed human platelets provides a stable reagent, easy to use, and one that permits replicate determinations to be made from day-to-day to month-to-month with great reproducibility if the same set of reagents (normal reference plasma, lyophilized venom coagglutinin, and lyophilized platelets) is used. In other studies, similar to those reported earlier with ristocetin,⁴ freeze-thawed or air-dried fixed platelets were used. While they behaved similarly to lyophilized platelets, we found the latter platelet reagent most convenient for storage and use. Apparently the glycoprotein complex in the platelet membrane, which is concerned with vWF-dependent platelet aggregation,^{8,9} maintains its functional activity essentially intact through the fixation and drying procedures.

The simple screening test with venom coagglutinin and lyophilized platelets for reduced vWF reported here appears to be a practical procedure for rapidly studying large numbers of plasmas. Either undiluted plasma or a diluted plasma is suitable (Table 1). The

availability of venom coagglutinin and lyophilized platelet reagents and the use of the macroscopic endpoint permit many plasma samples to be studied in a short time, similar to the efficiency of prothrombin time determinations with undiluted plasma. Ristocetin can also be used with the dried platelets for a vWF screening test.⁴

Bioassay methods for vWF using the new reagents were patterned after either the aggregometric¹⁰ or the macroscopic¹¹ test procedures with ristocetin. The venom coagglutinin may be employed over a wide

range of concentrations in the tests, unlike ristocetin, which has a narrow range suitable for vWF testing. Also, the venom coagglutinin is not a protein-precipitating agent and it does not cause the initial increase in opacity of the test mixture utilizing fixed or fixed dried platelets as observed with aggregometry if ristocetin is used.⁶

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