Functional Studies of Willebrand Factor Using Monoclonal Antibodies

By E. J. W. Bowie, D. N. Fass, and J. A. Katzmann

Previously described monoclonal antibodies to porcine Willebrand factor were used to study various functional assays of Willebrand factor. These antibodies comprise at least five separate specificities as determined by differential reactivity in three distinct binding assays. The antibodies were tested for their effect on the in vitro bleeding time, in vitro bleeding time, and ristocetin cofactor activity. The titers of the antibodies in a binding assay did not correlate with any inhibitory activities, and the ability to inhibit a given functional test was independent of inhibitory activity in the other assays. These data suggest that the antibodies are able to detect specific structures on Willebrand factor involved in specific functional assays and that the structure(s) involved in ristocetin cofactor activity is not identical to those mediating the in vivo or in vitro bleeding time.

Willebrand factor is a multimeric protein that circulates in the plasma as a series of high molecular weight polymers. The protein appears to be synthesized in the endothelial cell, and perhaps the megakaryocyte, and is also present in the platelet. The level of Willebrand factor in the plasma appears to play an important role in the physiology of hemostasis, particularly in the interaction of the platelet with the damaged blood vessel.

Platelets from patients with von Willebrand's disease adhere poorly to the de-endothelialized segments of rabbit aorta in the Baumgartner perfusion chamber. There is also defective adhesion of platelets to subendothelium at high shear rates when normal plasma is depleted of Willebrand factor by a specific antibody. The results of studies with the Baumgartner perfusion chamber using human renal arteries suggest that platelet adhesion is induced by Willebrand factor bound to the subendothelium and that it is the only plasma protein that is necessary for normal platelet adhesion.

Studies in our own laboratory using porcine tissues are entirely consistent with these results. Using a technique known as the in vitro bleeding time, it was found that normal blood or platelet-rich plasma produces hemostasis when allowed to flow through an incision in an excised piece of porcine skin. The incision in the skin was occluded at the epidermal end by a platelet clump that stained positively for Willebrand factor by immunofluorescence. Hemostasis did not occur with blood or platelet-rich plasma from a pig with von Willebrand's disease, unless Willebrand factor was added. The addition to normal pig blood of a polyclonal rabbit antiserum to Willebrand factor prolonged the in vitro bleeding time.

Similar results were obtained when plasma or concentrates of Willebrand factor were transfused into the living von Willebrand pig. The results were evaluated using the ratio bleeding time, which compares the blood loss in the first 3 min with the blood loss in the subsequent 12 min. When cryoprecipitate or a partially purified preparation of Willebrand factor was injected into a pig with severe von Willebrand's disease, the ratio bleeding time became normal. Other evidence suggests that the Willebrand factor in the platelet and the endothelial cell may also have a role to play in hemostasis, but is less important than the Willebrand factor in the plasma. It seems likely that the plasmatic Willebrand factor coats surfaces and serves as an attachment protein for the platelet.

We have previously reported on the production of hybridoma monoclonal antibodies to porcine Willebrand factor. The object of the investigations described in this article was to use the monoclonal antibodies as active-site modification reagents to see if the functions of Willebrand factor in hemostasis can be specifically and independently modified. Seven antibodies were tested for their effect on the in vivo ear bleeding time and the in vitro bleeding time. The results of these functional studies form the basis for this report.

MATERIALS AND METHODS

The von Willebrand Pig Colony

For several years, we have maintained a breeding colony of pigs with von Willebrand's disease. These animals share the impairment of primary hemostasis and other hemostatic abnormalities of the severe form of the disease in humans. The animals have a serious bleeding tendency, which is transmitted as an autosomal recessive characteristic. The abnormal tests of hemostasis include prolongation of the bleeding time, reduced platelet retention, almost complete absence of factor-VIII-related antigen (0.25% of normal), and the lack of ristocetin cofactor in the plasma.
Definition of Terms

**VIII:C** (VIII:coagulant) refers to factor VIII coagulant activity measured by the degree of correction of the clotting of human hemophilia A plasma, as measured by the activated partial thromboplastin time.\(^{23}\) Values above 1% of normal can be reliably measured for this factor. One percent of pig factor VIII:C activity is equivalent to approximately 8% human VIII:C.\(^{19}\)

**VIII:R:AG** (VIII-related antigen) refers to the antigenic activity related to factor VIII detected by a rabbit antibody to purified Willebrand factor,\(^{26}\) measured by electroimmunoassay.\(^{27}\) We calibrate this assay to a lower limit of 3%.

**VIII:R:WF** (VIII-related Willebrand factor, ristocetin cofactor) refers to the plasmatic activity that allows ristocetin to induce platelet aggregation. This activity is missing or reduced in the plasma in von Willebrand's disease. VIII:R:WF is measured by the degree of aggregation of washed gel-filtered human platelets using Olson's modification\(^{28}\) of Weiss' method.\(^{29}\) The standard curve for this assay extends from 6.25% to 50%. Values as low as 3% were measured by extrapolation.

Monoclonal Antibodies

The monoclonal antibodies to Willebrand factor that were used in these studies are fully described by Katzmann et al.\(^{18}\) The antibodies were originally identified by a radioimmunoassay based on the ability of immobilized hybridoma antibodies to bind porcine Willebrand factor as detected by the subsequent binding of an affinity-purified polyclonal rabbit anti-Willebrand factor labeled with \(^{125}\)I.\(^{121}\) The antibodies were subclassified according to their ability to react with immobilized porcine Willebrand factor or human Willebrand factor, to inhibit the platelet-aggregating activity of porcine Willebrand factor, and to inhibit ristocetin-induced platelet agglutination.\(^{14}\)

For each hybridoma line, ascites fluids were obtained in the absence of anticoagulant, and the harvested fluids were clarified by centrifugation. The fluids for an individual hybridoma were pooled, aliquoted, and frozen. The antibodies were titrated by determining the dilution of ascites fluid at which 50% binding of Willebrand factor occurred. One percent of pig factor VIII:C activity is equivalent to approximately 8% human VIII:C.\(^{19}\)

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**Polyclonal Antibody**

The polyclonal antibody to Willebrand factor was raised in rabbits against a purified preparation made by the technique of Olson et al.\(^{16}\)

### Table 1. Titration of Anti-Willebrand-Factor Ascitic Fluids

<table>
<thead>
<tr>
<th>Group</th>
<th>Clone*</th>
<th>RIA Titre†</th>
<th>Detects VIII:R:AG‡ Bound to Surface</th>
<th>Detects§ Human VIII:R:AG</th>
<th>Inhibits PAF and VIII:R:WF</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>W-1</td>
<td>8 x 10^11</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>W-1-8</td>
<td>1 x 10^8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>W-1-5</td>
<td>5 x 10^10</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>W-1-4</td>
<td>8 x 10^7</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>W-1-3</td>
<td>3 x 10^10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>W-1-23</td>
<td>5 x 10^11</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VII</td>
<td>W-1-2</td>
<td>8 x 10^9</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Clone W-1-4 was IgG<sub>2</sub>, and all others were IgG<sub>1</sub>, as determined by double diffusion immunoprecipitation and immunoelectrophoresis.
†Dilution at which 50% of maximum binding occurs.
‡All monoclonal antibodies detected VIII:R:AG in its soluble form; only those scored + will detect VIII:R:AG adsorbed to polystyrene.\(^{18}\)
§Katzmann et al.\(^{18}\)
∥Ristocetin-independent aggregation of human platelets by porcine or bovine VIII:R:WF.\(^{32}\)

The antibody gave a single precipitin line on immunodiffusion against normal pig plasma and was not reactive with plasma from a pig with severe von Willebrand's disease.

**Bleeding Times**

*The ear bleeding time* was measured by Mertz's modification\(^{30}\) of the immersion method of Doettl and Ripke.\(^{31}\) The ear was cleaned and shaved and an incision was made through the ear at the margin near the apex using a no. 11 Bard-Parker blade. The extent of the incision was limited by a cork stopper held on the posterior surface of the ear. The ear was immediately placed in a beaker containing 50 ml of isotonic saline solution, which had been warmed to 37°C, and the time of bleeding was measured. The bleeding time was independent of the volume of blood loss, as shown in Fig. 2, in which the relative volume of blood was measured by performing a red cell count on the blood collected into the saline during the first 3 min.

The in vivo bleeding time test was performed on normal Babcock pigs with an average weight of 4.9 kg (n = 14). The data did not disclose any effect of the small variations in the weight of the pigs on the apparent efficacy of the antibodies in prolonging the bleeding time. An initial preinjection bleeding time was performed on each ear. Ascitic fluids (0.2-0.4 ml) were then injected intravenously. Bleeding times were performed immediately after injection and at intervals of 5 min, 30 min, 1 hr, 2 hr, 4 hr, and 24 hr. At the same intervals, blood samples were withdrawn to be measured for VIII:C, VIII:R:AG, and VIII:R:WF.

*The in vitro bleeding time*\(^{34}\) is a technique developed in our laboratory in which heparinized normal blood or platelet-rich
In Vitro Bleeding Time

The antibodies were assessed for their ability to prolong the in vitro bleeding time. Figure 3 shows an example of the effect of two antibodies, one of which (W1-5) causes prolongation of the in vitro bleeding time and the other (W1-4) does not. All seven antibodies were measured at various dilutions in this test, and an attempt was made to rank the antibodies for potency according to their effect (Table 2). Antibodies 1, 2, and 8 showed inhibition of platelet plug formation at higher dilutions than the others and were considered to be the most potent. Conversely, antibody W1-4 showed no effect on platelet plug formation at the lowest dilution and was clearly the least potent.

Ear Bleeding Time

The antibodies were also assessed for their ability to prolong the ear immersion bleeding time (Table 2). Figure 4 shows an example of a series of experiments using antibody W1-8. Various antibody doses were injected into separate animals, and there was a dose-dependent prolongation of the ear bleeding time. The antibody in a dose of 0.40 ml caused a transient prolongation of the bleeding time to 14 min; 0.24 ml caused less prolongation, and 0.20 ml had no effect.

At each time a bleeding time was performed, a sample of plasma was obtained and assayed for VIII:C, VIIIIR:AG, and VIIIIR:WF. Even when antibody
resulted in prolongation of the bleeding time, the VIII:C, VIII:AG, and VIII:WF remained at the normal range. A typical experiment is shown in Fig. 5.

The in vivo bleeding time data for the antibodies at 5 min after injection are summarized in Table 2. These data represent the average of the bleeding time at 5 min postinjection obtained from both ears of either 2 or 3 separate animals injected with 0.4 ml ascites fluid. Antibody Wl-8 prolonged the bleeding time to more than 15 min and was clearly the most potent. Antibodies Wl-1 and Wl-3, like the saline control, caused no prolongation of the bleeding time and were the least potent. The remaining 4 antibodies prolonged the bleeding time to between 5 and 8 min and were considered to be of intermediate potency.

It is of interest that, with five of the antibodies, bleeding recurred from the incision in the ear after the initial cessation. We called this phenomenon "rebleeding."

DISCUSSION

The titration of the monoclonal antibodies by means of the radioimmunoassay allows their functional effects in different systems to be compared relative to their binding titer. The results of these studies show that the antibodies have different potencies in the functional tests employed. Antibody Wl-1, for example, which was one of the most potent in the in vitro bleeding time, was one of the least potent in the in vivo bleeding time. Antibody Wl-8, which was one of the least potent in the binding assay, was most potent in the in vivo and in vitro bleeding time.

Other similar contrasting observations can be made. Antibody Wl-2 inhibited ristocetin cofactor activity as well as the in vitro bleeding time and partially inhibited the in vivo bleeding time activity. This suggests that the structures mediating these three functional assessments are closely related. It should be noted, however, that other antibodies had no effects on ristocetin cofactor activity, but had similar or more potent effects on the bleeding times than antibody Wl-2. These data suggest that, whereas the structures mediating ristocetin cofactor activity, in vivo bleeding time, and in vitro bleeding time are closely related, they are distinct.

The ear bleeding time can be considered to be a measure of primary hemostasis, and the data we have presented show that some of the antibodies interfered with this mechanism. For the sake of clarity, we have only presented the data on the bleeding times that are obtained 5 min after injection of the antibody. On most occasions the bleeding time subsequently became normal, but in several instances, it again became prolonged. The prolongation usually occurred within 2 hr after the injection, but on 3 occasions, it was noted as long as 24 hr. On frequent occasions, it was also noted that after hemostasis had occurred, the wound started
The antibodies that cause this phenomenon are those that do not interact or interfere with other selected VIII-related activities.

Experiments have not yet been performed with Fab fragments, and it is therefore not directly known if crosslinking effects are responsible for the differential changes in function. It is unlikely that a single generalized event such as crosslinking can explain these results, because the monoclonal antibodies differentially affect bleeding time assays as well as the function of VIII:C, VIIIR:AG, and VIIIR:WF. In addition, no immunoprecipitate was seen in vitro over a wide range of concentrations. It seems likely, therefore, that the antibodies are dissecting out single functional sites, and although the data cannot be interpreted at a molecular level, these systems may be used to look specifically at various portions of the molecules and their various functions. This sort of approach can yield important information regarding the physiologic function of Willebrand factor and its role in platelet blood vessel interaction.

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REFERENCES

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