Effect of Monoclonal Antibodies Against von Willebrand Factor and Platelet Glycoproteins IIb/IIIa on the Platelet Retention Test

By Jean McPherson, Sandra Brownlee, and Marjorie B. Zucker

The platelet retention test provides a measure of the number of platelets retained in a column of glass beads and is one of the few in vitro platelet function tests that is abnormal in von Willebrand's disease (vWD). In a two-stage test, 1 mL of blood (designated A) was passed through the column, followed by 5 mL of isotonic saline and then 5 mL of blood (B) in which platelet retention was measured. With normal blood as A and B, retention is very high in all 5 mL of blood. In the first stage, platelets adhere to the glass beads; this requires fibrinogen but not von Willebrand factor (vWF). The platelet–platelet adhesion in the second stage requires vWF, is dependent on release of ADP, and fails to occur if thrombasthenic platelets are tested. Retention was normal when blood from a patient with afibrinogenemia was used as blood B. We have now used monoclonal antibodies to elucidate further the mechanism of platelet retention. Five antibodies to different epitopes on vWF essentially abolished retention in the one-stage test and in the second stage of the two-stage test, but had no effect on the first stage. Thus, the entire vWF molecule must be free of antibody to function in the platelet–platelet adhesion of the second stage of this test. Binding of the antigen–antibody complex to the platelet Fc receptor was not responsible, as Fab and F(ab')2 fragments of one of the antibodies were as effective as intact antibody, and as neither heat-aggregated IgG nor a polyclonal antibody to plasma factor IX inhibited retention. F(ab')2 fragments of 6D1, an antibody to platelet GP Ib that prevents binding of vWF to platelets, also inhibited the second phase of retention. An antibody that inhibits binding of fibrinogen and vWF to GP Ib/IIIa (LJ-CP8) inhibited both the first and second stages of retention, whereas LJ-PS, an antibody that inhibits only the binding of vWF to GP Ib/IIIa, caused slight inhibition of retention when normal or afibrinogenemic blood was used as blood B and was reported to cause only partial inhibition of ADP-induced platelet aggregation in this afibrinogenemic patient. The results suggest that vWF is altered during rapid passage of blood through the glass-bead column so that it attaches to GP Ib, exposing GP IIb/IIIa, which then binds the altered vWF or fibrinogen, either of which can induce platelet aggregation (platelet–platelet adhesion) and thus retention in the column.

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Platelet retention test. Columns were prepared as previously described,44 using 2 g of glass beads (diameter 0.45 to 0.5 mm) (Bacto Laboratories, Sydney, or 3M, Minneapolis), packed tightly into polyvinyl tubing, 3.5-mm ID. Columns were stored in a dessicator for at least 24 hours prior to use.

Materials and Methods

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Healthy normal subjects who had not taken aspirin or related drugs for at least 1 week were used as donors, as well as a previously studied patient with afibrinogenemia.\(^1,2\) (fibrinogen 3 to 10 µg/mL plasma; 5 µg/10\(^9\) platelets). Blood was collected through a 19-gauge butterfly infusion set into 30- or 35-ml syringes containing sodium heparin (Weddel Pharmaceuticals, Sydney, or Upjohn, Kalamazoo, MI; final concentration 4 U/mL). Care was taken to avoid air bubbles, and the contents were mixed by "twirling." These precautions to avoid disturbance of blood were previously described.\(^4\)

In some studies, an appropriate volume of either monoclonal antibody diluted in isotonic saline or sodium azide at a concentration equal to that in the antibody dilution was added. Antibody concentrations are given as microgram per milliliter of plasma, calculated by use of the hematocrit. Unless otherwise noted, antibodies were tested on at least three donors, with similar results. Figures 1 through 3 show typical experiments. Concentrations of azide up to 0.25 mmol/L in plasma had no effect on platelet retention.

Retention was measured between 10 and 40 minutes after blood collection. The syringe was twirled to mix the contents and placed in the holder of a perfusion pump (Harvard Apparatus, S Natick, MA). Blood was passed through the column at a speed of 6 or 8 mL/min, and each emerging milliliter was collected in 10 µL of 20% dipotassium EDTA. The microhematocrit was measured, PRP was separated in a Thrombocyte (Coulter Electronics, Hialeah, FL) or by sedimentation, and platelets were counted in a Coulter Model ZF or B. The percentage of retained platelets in each milliliter was calculated.

For the two-stage test, 1 mL of blood (A) was pumped into the column, followed rapidly by 5 mL of isotonic saline to flush the column. Then blood B was pumped through; the first milliliter, which was mostly saline, was discarded, and the platelets were counted in each of the succeeding 5 mL.

Monoclonal antibodies and other reagents. Dr. J.J. Sixma provided antibodies to vWF prepared by Dr. J.A. van Mourik as follows: RAg-35, RAg 38, and RAg 201 as IgG, and RAg Asc as ascitic fluid. Dr. D. Meyer provided antibody vWF 9 from which we prepared Fab' and F(ab')\(^2\) fragments by methods previously described.\(^1,2\) References and descriptions of the effects of these antibodies are given in Table 1. Dr. Z.M. Ruggeri sent us Fab' fragments of antibodies LJ-CP8 and LJ-PS that react with the platelet glycoprotein complex IIb/IIIa.\(^1,2\) Dr. B.S. Coller gave us antibody vWF 9 from which we prepared Fab and F(ab')\(^2\) fragments by methods previously described. Antibodies to vWF prepared by Dr. iA. van Mourik as follows: RAg 201 and RAg Asc had the same effect as RAg 38. Antibody vWF 9 from Meyer also inhibited platelet retention when added at concentrations of 3.2 to 3.6 µg/mL. At a concentration of 1.2 µg/mL, RAg-35 caused total inhibition of retention and the other three antibodies caused only partial inhibition (Fig 1A). In the two-stage test, none had any effect when added to blood A at 5 µg/mL, but all of them caused marked inhibition when added to blood B at 3.2 to 3.5 µg/mL (Fig 1B). Antibody vWF 9 from Meyer also inhibited the one-stage test in a dose-dependent manner, with a maximal effect at 5 µg/mL (Fig 1C). As with the Sixma antibodies, vWF 9 inhibited the two-stage test when added to blood B at 1 to 5 µg/mL, but was not effective when added to blood A at 5 µg/mL (not shown). Fab and F(ab')\(^2\) fragments of vWF 9 were as effective as whole IgG.

Polyclonal antibody to factor IX at concentrations up to 200 µg/mL and heat-aggregated IgG at concentrations up to 500 µg/mL had no effect on retention when used in a one-stage test or as blood A or B in the two-stage test.

F(ab')\(^2\) fragments of 6D1 at 10.5 µg/mL inhibited retention in the one-stage test in one donor (Fig 2A). In two other donors, they had no effect when added to blood A in the two-stage test (Fig 2B), but when added to blood B, inhibited...
Effective concentrations (Fig 2B) or nearly completely (7.5 µg/mL) caused retention partially (10.9 µg/mL, Fig 2B) or nearly completely (7.5 µg/mL, not shown).

Fab' fragments of antibody LJ-CP8 abolished retention when used at a concentration of 45 µg/mL in a one-stage test (Fig 3A) or as blood A or B in a two-stage test (Fig 3B). In the one-stage test, Fab' fragments of antibody LJ-P5 at 182 µg/mL caused very slight inhibition (Fig 3A); in other tests (not shown), however, retention was reduced by 16% to 20% with antibody concentrations of 86 and 169 µg/mL. Antibody had no effect when added to blood A and caused only minor inhibition when concentrations of 86 or 169 µg/mL were used in blood B (Fig 3C). When a fibrinogenemic blood was used as blood B in the two-stage test, retention was as high as in normal blood, but antibody LJ-P5 (133 µg/mL) caused slightly more inhibition than it did in normal blood (Fig 3D).

**Discussion**

The five monoclonal antibodies to vWF that we tested have different effects on the diverse functions of this large molecule (Table 1). They all inhibit platelet retention when added at low concentrations, however, including RAg Asc, which fails to affect any other activity of vWF (J.J. Sixma, personal communication). The antibodies affect only the second stage (blood B) of the two-stage test, which is the stage that requires vWF. Ogata and colleagues described four mouse monoclonal antibodies to vWF that inhibit platelet retention; only one of these inhibited ristocetin-induced agglutination.

Three observations provide evidence against the possibility that this lack of a differential effect resulted from nonspecific binding of antigen (i.e., vWF)–antibody complexes to the platelet Fc receptor, known to be closely associated with GP Ib. (a) Fab and F(ab')2 fragments of antibody vWF 9 inhibited retention as effectively as the whole antibody; (b) a polyclonal antibody to factor IX, a protein present in plasma at about the same concentration as vWF, failed to inhibit platelet retention; and (c) heat-aggregated IgG, which reacts with the platelet Fc receptor in the absence of plasma, had no effect on retention even at a very high concentration.

We and other researchers showed that antibody 6D1, directed against GP Ib, inhibits platelet retention. The dose–response relationship is not clear-cut, however. Coller and co-workers showed a reduction of retention from 95% to 48% in a one-stage test; greater inhibition could not be achieved by increasing the antibody concentration. We observed more marked inhibition (Fig 2A).

Although asialo vWF and vWF from patients with type IIB vWD bind to GP Ib without addition of ristocetin, normal vWF binds to GP Ib only in the presence of ristocetin. Interaction of all three forms of vWF with platelets is prevented by monoclonal antibodies to GP Ib. The requirement for GP Ib in platelet retention suggests that passage of blood through the glass-bead column at high shear rate may alter either vWF or GP Ib in a way that results in their association. The observation that any of five monoclonal antibodies to different epitopes on vWF inhibits retention suggests that their attachment may prevent an alteration of vWF. Of interest is the recent report of a kindred with a variant form of vWD in which platelet retention was the only abnormal test. Plasma contained vWF that had retained the "pro" piece (ref. 33; T.S. Zimmerman, personal communication). These heterozygous subjects did not have a hemorrhagic diathesis.

Antibody LJ-CP8, which, like LJ-CP9, prevents binding of fibrinogen and vWF to the GP Ib/IIIa of activated platelets (Z.M. Ruggeri, personal communication), caused marked inhibition of both stages of retention. This is in accord with the abnormality of both stages of retention in thrombasthenic blood and the inhibitory effect on the one-stage test of antibody 10E5 against GP Ib/IIIa.

Antibody LJ-P5 inhibits binding of vWF but not of fibrinogen to GP Ib/IIIa on activated platelets. This antibody had only a slight inhibitory effect on the one-stage and two-stage tests in normal blood. According to De Marco and

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**Table 1. In Vitro Effects of Monoclonal Antibodies Against vWF**

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<tr>
<th>Antibody</th>
<th>Designation</th>
<th>Source</th>
<th>Reference</th>
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<th>Conc. *</th>
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<td>7</td>
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NR, not reported.

*The inhibitory concentrations (Conc. Inhib.) of antibody are the lowest values given in the original articles but are not necessarily the minimum effective concentrations.

†Data from present study. Antibody concentrations represent lowest effective plasma concentration.

‡This antibody binds to vWF but does not affect its function (J.J. Sixma, personal communication).

§Fab and F(ab')2 fragments were equally inhibitory.
colleagues,14 LJP5 has no effect on ADP-induced aggregation in normal citrated PRP but abolishes the moderate platelet aggregation that occurs in the citrated PRP of afibrinogenemic patients with plasma fibrinogen concentrations of <8.5 μg/mL. ADP-induced aggregation in these afibrinogenemic patients was increased by addition of vWF and was believed to be mediated at least in part by binding of vWF to GP IIb/IIIa; this binding does not occur in the presence of physiologic concentrations of fibrinogen.34,35 In our patient, ADP-induced aggregation was subnormal in the presence of physiologic concentrations of fibrinogen.34,35 In agreement with our earlier findings,12 retention was normal when his blood was used as blood B. Although LJP5 caused more inhibition than it did in normal blood when it was added to blood B, it was still slight, suggesting that the patient’s fibrinogen concentration (3 to 10 g/L) may be sufficient to support platelet–platelet adhesion in the second stage of platelet retention. This is supported by the finding that LJP5 did not completely inhibit primary ADP-induced platelet aggregation in citrated PRP from this patient (H.J. Weiss, personal communication).

vWF, but not fibrinogen, is essential for platelet aggregation in citrated blood or PRP that has been subjected to high shear stress in a cone and plate viscometer.14 vWF also plays a part in the formation of thrombi in native blood perfused at low shear rate over aortic endothelium36,37 and in the formation of occlusive thrombi in damaged coronary arteries in pigs.38 Thus, events in a glass-bead column are relevant to important physiologic and pathologic processes. The mechanism by which vWF promotes platelet–platelet adhesion is not yet understood. Conditions in the glass-bead column resemble those in the circulation more closely than conditions in the usual in vitro studies of platelets, as the studies are carried out in whole blood with normal divalent cation concentration, and the disturbance caused by centrifugation is avoided. We hope that further studies will clarify the mechanisms.

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