A monoclonal antibody directed against the von Willebrand factor moiety (vWF) of factor VIII-von Willebrand factor (FVIII-vWF), which blocks ristocetin-induced platelet aggregation as well as the binding of FVIII-vWF to platelets in the presence of ristocetin, inhibited platelet adherence to human artery subendothelium when present in normal flowing blood. This monoclonal antibody, CLB-RAG 35, inhibited platelet adherence as a function of the shear rate. At wall shear rates below 500 s⁻¹, platelet adherence was not affected, but at higher shear rates platelet adherence was gradually inhibited, reaching an average of 11% of the normal value at 2,500 s⁻¹. Indirect immunofluorescence established the reactivity of CLB-RAG 35 with vWF present in artery subendothelium. Pretreatment of normal vessel walls with this antibody inhibited adherence of platelets in blood from a patient with severe homozygous von Willebrand’s disease and in blood from normal individuals. The inhibition was shear-rate dependent and significant at high shear rates (2,500 s⁻¹). By adding increasing amounts of purified FVIII-vWF to normal blood, the inhibition was gradually overcome. These data indicate that vWF present in the vessel wall contributes appreciably to platelet adherence. At high wall shear rates, platelet adherence is mediated virtually completely by both plasma FVIII-vWF and vWF in the vessel wall. At low wall shear rates (below 500 s⁻¹), platelet adherence occurs independent of FVIII-vWF in plasma and vWF in the vessel wall.

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PLATELET ADHERENCE at the site of vascular injury is an important step in the early phase of hemostasis,¹ thrombogenesis,² and atherogenesis.³ These observations have spurred on the interest in factors involved in platelet adherence.

Tschop and associates⁴ have shown that platelet adherence to rabbit aorta subendothelium in laminar-flowing blood from patients with von Willebrand’s disease (vWD) was impaired when compared with platelet adherence in normal blood. This abnormality was more pronounced at high wall shear rates⁵ and could be corrected by adding cryoprecipitate.⁶ Shear-rate–dependent impairment of platelet adherence was also observed when antibodies directed against factor VIII-von Willebrand factor (FVIII-vWF) were added to normal blood.⁷⁸

Experiments with reconstituted blood have shown that FVIII-vWF is the only plasma factor that mediates platelet adherence to human artery subendothelium.⁹ When plasma FVIII-vWF was bound to subendothelium and subsequently exposed to reconstituted blood, platelet adherence was positively correlated to the amount of bound FVIII-vWF.⁹ Time-sequence studies suggested that binding of FVIII-vWF to the subendothelium precedes platelet adherence.¹⁰ In these studies, however, platelet adherence in plasma or plasma substitutes deficient in FVIII-vWF was still about 50% of the adherence level in normal plasma. It has been suggested but not proved that platelet adherence in the absence of plasma FVIII-vWF is mediated by von Willebrand factor (vWF) already present in the subendothelium of the vessel wall.¹¹¹²

To investigate the contribution of plasma FVIII-vWF and pre-existing subendothelial vWF in mediating platelet adherence, we have used a monoclonal antibody directed to the vWF moiety of FVIII-vWF that blocks ristocetin-induced platelet aggregation and prevents the binding of FVIII-vWF to platelets. Platelet adherence was virtually completely inhibited by this antibody at high shear rates but not at low shear rates. When vessel segments were preincubated with this antibody, subsequent platelet adherence was inhibited. These findings demonstrate a significant contribution of vessel wall vWF to platelet adherence.

MATERIALS AND METHODS

Materials

All chemicals purchased from commercial sources were of the highest purity available (J.T. Baker Chemicals BV, Deventer, The Netherlands; British Drug House Chemicals Ltd, Poole, UK; E. Merck, Darmstadt, West Germany, and Fluka AG, Buchs SG, Switzerland). Ristocetin was obtained from H. Lundbeck & Co A/S (Copenhagen, Denmark), ¹³¹⁴In-oxine was obtained from Byk Mal- linckrodt (Petten, The Netherlands), and fluorescein-isothiocyanate (FITC)–labeled goat anti-mouse immunoglobulin was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands).

From the Department of Blood Coagulation, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, and the Department of Haematology, State University Utrecht, Utrecht, The Netherlands.

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Blood Collection and Measurements of FVIII-vWF–Related Activities

Blood from eight normal individuals and from one patient with homozygous severe vWD was Anticoagulated with 1/10 vol 110 mmol/L trisodium citrate following a clean venepuncture with a 1.2-mm-Ø needle (Strauss Kanüle, Süd-Deutsche Feinmechanik, Wächtersbach, West Germany). The blood was stored for 30 minutes at 22 °C before it was used for the perfusion experiments or for radiolabeling of blood platelets.8,10 The patient with homozygous severe vWD supplied blood on two occasions at an interval of four months.

The FVIII-vWF-related activities, ristocetin cofactor activity (FVIIIIR:RCF), FVIII-procoagulant activity (FVIII:C), and FVIII-related antigen (FVIIIIR:Ag), were determined as previously described.11,18 One unit of activity or antigen was defined as the amount present in 1.0 mL of pooled frozen citrated (135 mmol/L) plasma from 40 healthy donors. The plasma of the patient with vWD contained 0.01 unit of FVIII:C per milliliter, less than 0.01 unit FVIIIIR:Ag per milliliter, and less than 0.02 unit FVIIIIR:RCF per milliliter. The FVIII-vWF-related activities in the blood from the normal subjects were in the following range: 0.75–1.15 unit FVIII:C per milliliter, 0.85–1.45 unit FVIIIIR:Ag per milliliter, and 0.70–1.20 unit FVIIIIR:RCF per milliliter.

Platelet counts and hematocrit levels from all donors were measured in 0.4% EDTA blood with a Thrombo Counter and a Coulter Counter, model S (Coulter Electronics Ltd., Harpenden, UK). Platelet count and hematocrit were in the range of 2.0–2.5 × 10^11/L and 41.7–48.9%, respectively.

Purification of Plasma FVIII-vWF

FVIII-vWF was purified from cryoprecipitate of human plasma as described by Van Mourik and Mochtar17 with some minor modifications.8 When purified FVIII-vWF was used in perfusion experiments, the protein preparation was dialyzed for one hour against Michaelis buffer (0.0285 mol/L sodium acetate, 0.0285 mol/L sodium barbital, 0.116 mol/L NaCl, pH 7.35) before addition to the perfusate.

Monoclonal Antibodies to vWF

In the study reported here, we used monoclonal antibodies coded CLB-RAg 35 and CLB-RAg 50. These two antibodies, which are directed to different epitopes on vWF, have been described in detail elsewhere.8,19

Ristocetin-Induced Binding of FVIII-vWF to Platelets in the Presence of CLB-RAg 35 and CLB-RAg 50

Human platelets were washed according to Sakariassen et al9 and adjusted to 10^11 platelets per liter. Platelet-poor plasma (200 μL), obtained from a healthy volunteer, was incubated with 0.2 μg monoclonal IgG (10 μL). After a 30-minute incubation at room temperature, aliquots of 50 μL were distributed to microcentrifuge tubes. Platelets (20 μL) and ristocetin (5 μL, 1.5 mg/mL final concentration) were added, and the suspension was incubated for 30 minutes without stirring. After having centrifuged the samples (12,000 g, two minutes at 22 °C), the supernatant plasma was assayed for FVIIIIR:Ag with an immunoradiometric assay using heterologous antibodies to vWF and pooled normal plasma as standard.20

Perfusion Studies

Five-minute perfusions with whole blood or reconstituted blood were carried out with human de-endothelialized umbilical arteries21,22 at 37 °C in annular perfusion chambers as described by Baumgartner.23 To obtain wall shear rates of 1,000 s^-1, 1,800 s^-1, and 2,500 s^-1, a recently developed small annular perfusion chamber with an effective annular width of 0.60 mm was used.24 The annular wall is the distance between the chamber wall and the central rod of the perfusion chamber minus the average thickness of the artery wall (∼0.15 mm). The standard chamber with an effective annular width of 1.05 mm was used to obtain wall-shear rates of 300 s^-1 and 500 s^-1. Perfusates (whole blood from normal subjects and from the patient with vWD) were incubated for ten minutes at 37 °C with monoclonal antibodies (10 μL ascites per milliliter of blood) or with purified FVIII-vWF (final concentration of 2.85 units and 3.85 units FVIIIIR:RCF per milliliter, respectively) before a perfusion run. Control perfusates were similarly treated, but without addition of monoclonal antibodies and purified FVIII-vWF, respectively.

Vessel segments (length of 10 mm) were pretreated by (static) incubation with ascites CLB-RAg 35 and CLB-RAg 50 at 37 °C for 45 minutes in glass tubes, with the artery segment mounted on the central rod of the perfusion chamber. The ascitic fluids were diluted 100 times in 0.2 mol/L Krebs-Ringer buffer (4 mmol/L KCl, 107 mmol/L NaCl, 20 mmol/L NaHCO3, 2 mmol/L Na2SO4) containing 19 mmol/L trisodium citrate and 2.5 mmol/L CaCl2 at pH 7.35. For control experiments, vessel segments were exposed to buffer alone, without monoclonal antibodies. Before perfusion with blood, the pretreated vessels were rinsed by perfusion with 100 mL of 0.2 mol/L TRIS-HCI buffer (pH 7.35). In another set of control experiments, two perfusion chambers were connected in series. In one perfusion chamber, the artery segment was pretreated with CLB-RAg 35, and a nontreated artery segment was placed in the other perfusion chamber.

Platelet-subendothelium interaction was measured by morphometric evaluation25 or by using 11C-in-radiolabeled blood platelets.26 Previously, we have shown that the radiolabeling method correlates well with the morphometric method.19

Platelet adherence (percentage of adherence) was scored according to the method of Baumgartner et al27 as the sum of the percentage of contact and the percentage of spread platelets covering the subendothelium. Aggregate formation was defined as the total percentage of spread platelets covered with more than three platelets in height.

Immunoﬂuorescence Studies

For immunoﬂuorescence studies, segments of human umbilical arteries, mounted on rods of the perfusion chamber, were incubated for 45 minutes at 37 °C with ascites CLB-RAg 35 and CLB-RAg 50, diluted 100 times in 0.2 mol/L Krebs-Ringer buffer (pH 7.35). In a control experiment, a vessel segment was exposed to a monoclonal antibody directed against cat allergen (a gift from Dr R.C. Aalberse, CB, Amsterdam, The Netherlands). After 45 minutes' incubation at 37 °C, vessel segments were washed twice with 0.2 mol/L TRIS-HCI buffer (pH 7.35), and frozen in liquid nitrogen either directly or after a five-minute perfusion of the vessel segments with perfusates deficient in FVIII-vWF. Cryostat sections of 5-μm thickness were air-dried, fixed for 10 minutes in acetone, and washed with 0.15 mol/L phosphate-buffered saline (PBS), pH 7.4. The sections were incubated for one hour with FITC-labeled goat anti-mouse immunoglobulin (diluted 1:80 in PBS), washed with PBS, embedded in PBS containing 10% glycerol, and examined with a fluorescence microscope (Leitz, Wetzlar, West Germany).
RESULTS

Effect of Monoclonal Antibodies on Ristocetin-Induced Binding of FVIII-vWF to Platelets

As shown in Table 1, CLB-RAg 35, at a concentration of 0.6 μg IgG/mL, completely inhibited binding of FVIII-vWF to platelets. CLB-RAg 50, at the same concentration, did not inhibit binding of FVIII-vWF to platelets.

Effect of CLB-RAg 35 and CLB-RAg 50 on Platelet Adherence at Various Wall Shear Rates

Shear-rate-dependent inhibition of platelet adherence was observed when CLB-RAg 35 was added to blood from normal subjects (Fig 1). At wall shear rates exceeding 1,000 s⁻¹, platelet adherence was gradually impaired, and it dropped to an average value of 47% and 11% of the normal values at 1,000 s⁻¹ and 2,500 s⁻¹, respectively. At lower wall shear rates, 300 s⁻¹ and 500 s⁻¹, platelet adherence was not affected by CLB-RAg 35.

Platelet aggregate formation on the subendothelium was not significantly influenced by CLB-RAg 35 at any wall shear rate. CLB-RAg 50 affected neither platelet adherence nor aggregate formation (data not shown).

Effect of CLB-RAg 35 and CLB-RAg 50 on Platelet Adherence in Blood of a Patient With vWD

At a shear rate of 2,500 s⁻¹, platelet adherence in blood from the patient with vWD was strongly impaired (Fig 2). Addition of CLB-RAg 35 to the patient’s blood did not significantly affect platelet adherence. The level that was found was in the same range as observed with CLB-RAg 35 added to normal blood (Fig 2). CLB-RAg 50 did not affect platelet adherence in blood from the patient with vWD.

Aggregate formation in blood from the patient with vWD was not evaluated because too few platelets had spread out on the surface to study the interaction of other platelets with these spread platelets.

Effect of Pretreatment of the Vessel Wall

Pretreatment of artery subendothelium with CLB-RAg 35 inhibited platelet adherence in normal blood from an average value of 53% toward 23% and in blood from the patient with homozygous severe vWD from 15% to 3% at a wall shear rate of 2,500 s⁻¹ (Fig 3). At a wall shear rate of 300 s⁻¹, no inhibition of platelet adherence was observed (results not shown). Treat-

Table 1. Residual Amount of FVIII:Ag Following Ristocetin-Induced Binding of FVIII-vWF to Platelets: Effect of CLB-RAg 35 and CLB-RAg 50

<table>
<thead>
<tr>
<th>Platelet-Poor Plasma ± Monoclonal antibody (0.6 μg IgG/mL) + ristocetin (1.5 mg/mL)</th>
<th>Platelet Addition</th>
<th>Residual Amount of FVIII:Ag (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma alone</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Plasma alone +</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Plasma + CLB-RAg 35</td>
<td>–</td>
<td>93</td>
</tr>
<tr>
<td>Plasma + CLB-RAg 50</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Plasma + CLB-RAg 50</td>
<td>+</td>
<td>107</td>
</tr>
<tr>
<td>Plasma + CLB-RAg 50</td>
<td>+</td>
<td>14</td>
</tr>
</tbody>
</table>

After incubation, samples were centrifuged, and the supernatant solution assayed for FVIII:Ag with an immunoradiometric assay using heterologous antibodies (20) (mean of two experiments).
I0 - fl

Fig 4. Indirect immunofluorescence of vWF-related protein in subendothelium of human umbilical artery with CLB-RAg 35 (A) and CLB-RAg 50 (B). A monoclonal antibody directed against a cat allergen was used as control antibody (C). (Original magnification x400).

Immunofluorescence studies revealed binding of CLB-RAg 35 and CLB-RAg 50 to the subendothelium (Fig 4), both before and after five minutes of perfusion. Light microscopic examination of the vessel segments showed that no endothelial cells were present. No evidence of detachment of bound CLB-RAg 35 from the subendothelium during the perfusion was observed in studies with two perfusion chambers connected in series. The deposition of 111In-labeled platelets on the subendothelium of the vessel segment pretreated with CLB-RAg 35 was strongly inhibited, in contrast to the unaffected platelet deposition on the subendothelium of the nontreated control vessel segment in the second perfusion chamber.

Inhibition of platelet adherence, using normal blood, on vessel walls pretreated with CLB-RAg 35 could be overcome by the addition of purified FVIII-vWF to the perfusion fluid (Fig 5). Platelet adherence was almost normalized by addition of purified FVIII-vWF to a final concentration of 2.85 units FVIIIIR:RCF per milliliter.

In normal blood, aggregate formation on vessel walls treated with either CLB-RAg 35 or CLB-RAg 50 was not affected. Aggregate formation in blood from the patient with vWD was not evaluated because of the low percentage of spread platelets.

DISCUSSION

A monoclonal antibody directed against the vWF moiety of FVIII-vWF was used to investigate the relative contribution of plasma FVIII-vWF and vessel
wall vWF in mediating platelet adherence to artery subendothelium. This antibody, CLB-RAg 35, completely inhibited ristocetin-induced platelet aggregation and blocked the binding of FVIII-vWF to the platelet (Table 1). The complete inhibition of both phenomena, which could be achieved at picomolar concentrations, indicates that this antibody specifically prevents the interaction between platelets and FVIII-vWF.

As demonstrated by indirect immunofluorescence, the arterial subendothelium used in our studies was immunoreactive with CLB-RAg 35 before perfusion, indicating that the subendothelium contained vWF-related protein, probably deposited by endothelial cells. Vessel walls treated with CLB-RAg 35 before exposure to whole blood and to reconstituted blood showed inhibited platelet adherence at a high wall shear rate (2,500 s⁻¹, Fig 3), but not at a low wall shear rate (300 s⁻¹). The relative contribution of vessel wall vWF in mediating platelet adherence at 2,500 s⁻¹ under the experimental conditions was on the average 57% and 80% of the control values with blood from normal individuals and blood from a patient with homozygous severe vWD, respectively (Fig 3). Therefore, adherence of platelets in blood from patients with vWD to normal subendothelium is almost entirely mediated by vWF in the vessel wall. Preliminary data from Turrito et al also indicated that the vWF-related protein in the vessel wall plays a role in platelet adherence. The small residual platelet adherence of 3% observed at 2,500 s⁻¹ with vWD blood perfused over CLB-RAg 35 pretreated vessel segments (Fig 3) may have been caused by incomplete blocking of vessel wall vWF by CLB-RAg 35, or, alternatively, by interaction of platelets with other vessel wall components not mediated by vWF.

When normal blood is perfused over CLB-RAg 35--treated vessel segments, the residual platelet adherence can be explained by the interaction of platelets with plasma FVIII-vWF bound during the perfusion to the subendothelium. The inhibition of platelet adherence in normal blood by pretreatment of the vessel wall with CLB-RAg 35 may indicate that in normal plasma an insufficient amount of FVIII-vWF is present to completely replace the function of vWF in the subendothelium. This is corroborated by the fact that the inhibition of platelet adherence was corrected by addition of purified FVIII-vWF (Fig 5). The decrement in platelet adherence observed with CLB-RAg-35--treated vessel segments potentially could be the result of detachment of CLB-RAg-35 antibodies from the vessel wall and the appearance of the antibody in the perfusate. This possibility could be excluded, however, since perfusion experiments with two perfusion chambers in line, in which the first contained a CLB-RAg-35--pretreated vessel segment, did not show a decrement of platelet adherence on the nontreated vessel wall in the second perfusion chamber. We cannot rule out, however, that after incubation of the vessel segments with CLB-RAg 35 some antibody excess has remained in the subendothelium, which could partly block the function of subendothelium-bound plasma FVIII-vWF.

The inhibition of platelet adherence when CLB-RAg 35 was added to normal blood or when vessel walls were pretreated with CLB-RAg 35 was shear-rate-dependent and most pronounced at microcirculatory values exceeding 1,000 s⁻¹. At the highest shear rate used (2,500 s⁻¹), which is well within the physiologic range, platelet adherence could be inhibited by 89%, implying that under these conditions virtually all adherence is mediated by FVIII-vWF. In contrast, no inhibitory effect of this antibody was observed at low shear rates (300 s⁻¹), which corresponds to those observed in large veins. Therefore, platelet adherence to subendothelium occurs by at least two mechanisms. At high shear rates, platelet adherence is mediated virtually completely by both plasma FVIII-vWF and vWF present in the subendothelium. In contrast, at low shear rates, platelet adherence is not dependent on FVIII-vWF. This is in accord with the noninhibitory effect reported for homologous and heterologous antibodies against FVIII-vWF on platelet adherence to rabbit subendothelium at this shear rate.

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