Characterization of 25 Monoclonal Antibodies to Factor VIII-von Willebrand Factor: Relationship Between Ristocetin-Induced Platelet Aggregation and Platelet Adherence to Subendothelium


We have studied the role of factor VIII-von Willebrand factor (FVIII-vWF) in both platelet adherence to subendothelium and ristocetin-induced platelet aggregation using monoclonal antibodies to human FVIII-vWF. Twenty-five monoclonal antibodies were obtained, two of which were directed to the factor VIII moiety of FVIII-vWF; one of these two completely inhibited the procoagulant activity (FVIII:C). The remaining 23 monoclonal antibodies were directed to the von Willebrand factor moiety of FVIII-vWF. The ability of the latter monoclonal antibodies to inhibit platelet adherence to arterial subendothelium was investigated with a perfusion model. According to the number of platelets adhering to the subendothelium, three groups of monoclonal antibodies could be discerned: (A) antibodies not affecting platelet adherence; (B) antibodies that inhibited platelet adherence to the level as observed when von Willebrand's disease plasma was tested; and (C) antibodies that completely inhibited both platelet adherence to subendothelium and ristocetin-induced platelet aggregation. The two antibodies present in group C competed for the same or closely related epitope(s) present on FVIII-vWF. These results demonstrate that a domain is present on the FVIII-vWF molecule that is associated both with ristocetin-induced aggregation and with the ability of FVIII-vWF to support platelet adherence to the subendothelium. Based on these observations, it is concluded that ristocetin-induced binding of FVIII-vWF to platelets reflects, at least in part, a physiologic mechanism regulating the function of FVIII-vWF in primary hemostasis.

Factor VIII-von Willebrand Factor (FVIII-vWF), a high molecular weight glycoprotein complex that plays an important role in hemostasis, consists of two immunologically as well as functionally discernible moieties that can be isolated separately. These are the factor VIII moiety, which is associated with the factor VIII procoagulant activity (FVIII:C) and the factor VIII procoagulant antigen (FVIIIIC:Ag), and the von Willebrand factor moiety, which is associated with the factor VIII-related antigen (FVIIIIR:Ag). Hemophilia A is characterized by an abnormality of FVIII:C. Von Willebrand's disease is characterized by an abnormality of von Willebrand factor. (For a review about structure and function of FVIII-vWF that is associated both with ristocetin-induced aggregation and with the ability of FVIII-vWF to support platelet adherence to subendothelium, see Hoyer and Zimmerman et al.3)

The von Willebrand factor (vWF) is involved both in platelet–platelet interaction14 and in platelet adherence to subendothelium.3-7 In vitro, receptor-mediated binding of FVIII-vWF to platelets can be demonstrated in the presence of the antibiotic ristocetin.4 However, the physiologic significance of the ristocetin-induced FVIII-vWF platelet interaction is not clear. Furthermore, the interrelationship between ristocetin-induced platelet aggregation and the ability of FVIII-vWF to support platelet adherence to subendothelium has not been assessed.

Using the hybridoma technique,8 one may now obtain serologic reagents that are monospecific and, more importantly, are directed against single antigenic determinants present on FVIII-vWF. Therefore, it is possible to obtain antibodies directed against individual antigenic sites associated with unique functional activities.

In this article, we describe the characterization of 25 monoclonal antibodies directed against antigenic determinants located on FVIII-vWF. Two monoclonal antibodies, directed against the same epitope on the von Willebrand factor moiety of FVIII-vWF, completely inhibited the ristocetin-induced platelet aggregation as well as the ability of FVIII-vWF to support platelet adherence to subendothelium.

These results demonstrate that a domain is present on FVIII-vWF that is associated both with ristocetin cofactor activity (FVIIIIR:RCoF) and with the ability of FVIII-vWF to support platelet adherence to subendothelium.

MATERIALS AND METHODS

Monoclonal Antibodies

BALB/c mice were immunized with purified FVIII-vWF.9 Lymphocyte hybridization was performed by the method of Galfré et al.10 Description of the techniques used for screening and characterization of monoclonal antibodies directed against FVIII-vWF have been provided elsewhere and are applicable to this study.11 For the initial screening and preliminary characterization of the monoclonal antibodies, we used a purified FVIIIIC:Ag preparation12 that contained on average 5 U FVIII:C, 5 U FVIIIIC:Ag, and 0.001 U FVIIIIR:Ag/ml. Ascitic fluid, containing monoclonal antibodies, was used as the source of monoclonal antibodies.

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

Submitted May 16, 1983; accepted January 10, 1984.

No reprints are available.

© 1984 by Grune & Stratton, Inc.

0006-4971/84/6306-0022$03.00/0

Blood, Vol. 63, No. 6 (June), 1984: pp. 1408-1415
was produced by injecting the final cloned hybrid cells in pristane-
prived BALB/c mice. Titer (as tested with ELISA techniques) re-
ects the highest dilution at which ascitic fluids remained positive
(binding twice that of unrelated control ascitic fluid).

Monoclonal antibodies were purified from ascites by absorption to a
protein A-Sepharose column (Pharmacia, Uppsala, Sweden).13

125I-labeling of monoclonal antibodies was carried out by using the
iodogen reaction.14 Free 125I was removed by dialysis against phos-
phate-buffered saline (PBS). The radiolabeled monoclonal antibod-
ies were diluted in PBS-Tween 0.05%, which contained 3% bovine
albumin (BSA), before use in the assay.

**Inhibition of FVIIIR:RCof**

Inhibition of FVIIIR:RCof was tested in a system with freshly
washed platelets. Platelets were washed according to MacFarlane et
al.,15 and adjusted to a concentration of 200,000 platelets/μl. Sev-
enty-five microliters of 2.5 times diluted normal

Platelet–Subendothelial Interaction

Perfusions were carried out at 37°C in a perfusion chamber,16 as
developed by Baumgartner,17 with an effective annular width of 0.6
mm (the distance between chamber wall and the central rod, with
the average thickness of the artery wall) and a rod length of 72 mm.
Constant flow was obtained by gravity18 and was maintained at 96
ml/min (wall shear rate 2500 sec⁻¹). Subendothelium was obtained
from human umbilical arteries and exposed to perfusates made up
from washed 125I-labeled platelets (diluted to 1.2 x 10¹¹/liter), 40% red blood cells, and plasma from blood anticoagulated with 1/10 vol 100 mM trisodiumcitrate. Aspirin treatment of
platelets was performed simultaneously with the radiolabeling of the
platelets. This treatment prevents thrombus formation in citrated
blood on subendothelium.18 The 125I-labeled platelets were resus-
pended in autologous plasma, which had been preincubated for 30
min at room temperature with various monoclonal ascitic fluids (10
μl ascites/ml plasma). After a 5-min preincubation at 37°C, the
perfusates were recirculated for 5 min. Determination of adherent
platelets/sq cm subendothelium was performed as described.18

**Immunofluorescence**

For immunofluorescence on endothelial cells, these cells, derived
from human umbilical veins,19 were cultured on glass coverslips until
confluency was reached. After several washings with PBS to remove
traces of serum, endothelial cells were fixed by the addition of
methanol at room temperature for 10 min. Next, coverslips were
washed 3 times with PBS and incubated with monoclonal ascitic fluid
(diluted 1:1,000 in 0.2% gelatin in PBS) for 1 hr at ambient
temperature. After washing, as described above, the slides were
further exposed to fluorescein-isothiocyanate (FITC) labeled goat
anti-mouse immunoglobulin (CLB) (diluted 1:80 in 0.2% gelatin in
PBS). After 1 hr at room temperature in a moist chamber and 3
washings with PBS, coverslips were embedded in 10% glycerol in
PBS and examined with a fluorescence microscope.

For immunofluorescence studies on platelets, smears made from
platelet-rich plasma were air-dried and fixed in acetone at room
temperature for 10 min. After 3 washings with PBS, the smears were
incubated with antiserum as described above.

**Gel Electrophoresis and Immunoblotting**

Sodium dodecyl sulfate (SDS) agarose gel electrophoresis was
performed in 1.5-mm thick slab gels containing 1% agarose (Seakem
HGT, Rockland, Gilbertsville, PA), 0.19 M glycine, 30 mM Tris,
0.1% (w/v) SDS, pH 8.6. The stacking gel contained 1% (w/v)
agarose, 100 mM Tris-HCl, and 0.1% (w/v) SDS, pH 6.9. SDS-
agarose-polyacrylamide gel electrophoresis was performed in the
same buffer system. The separation gel consisted of 1% (w/v)
agarose, 4% (w/v) acrylamide, and 0.1% (w/v) bis-acrylamide.

Nonreduced samples were incubated for 15 min at 37°C in sample
buffer (final concentrations: 100 mM Tris-HCl, 2% SDS, 10%
glycerol, pH 6.9). Reduced samples were obtained by adding mer-
captoethanol to a final concentration of 2% (w/v), followed by a
15-min incubation at either 37°C or 100°C.

After gel electrophoresis, the proteins were transferred electropho-
retically to nitrocellulose filters (Schleicher and Schull, Dassel,
F.R.G.), essentially as described by Towbin et al.20 The blots were
washed in a buffer containing 50 mM Tris-HCl, 150 mM NaCl,
BSA 1%, and Tween 0.05%, pH 8.6, and then incubated overnight
with antiserum (rabbit antiserum against FVIII-vWF or monoclonal
ascitic fluid) diluted in the same buffer. After washing, the filters
were labeled by incubation with either 125I-sheep anti-rabbit IgG or
125I-goat anti-mouse IgG (4 x 10⁶ cpm/sq cm). In other cases, the
filters were labeled directly with a 125I-labeled rabbit antiserum
against FVIII-vWF or with 125I-labeled monoclonal antibodies.

For immunofluorescence studies on platelets, autoradiography was
performed at −70°C on Kodak XRP film.

**Competitive Binding Studies**

Polystyrene tubes (7 x 50 mm) were coated with purified FVIII-
vWF (20 μg/ml in 0.02 M sodium phosphate buffer, pH 7.5,
incubation overnight at 4°C). The tubes were washed 3 times with
PBS-Tween 0.05% and then incubated with 125I-labeled monoclonal
 antibodies in the presence of PBS-Tween 0.05%–BSA 3% without
unlabeled monoclonal antibodies.

**RESULTS**

Three fusions were performed on the spleen cells of 3
mice. After cloning and recombining, 25 hybridoma cell
lines were obtained. Table 1 shows the characterization
of the ascitic fluids from these 25 hybridoma cell
lines with regard to their immunologic and functional
properties. All 25 ascitic fluids bound to FVIII-vWF
purified from normal plasma and to FVIII-vWF puri-
ified from factor VIII concentrate (not shown). Twenty-
three ascitic fluids were positive with FVIII-vWF
present in normal pooled plasma and with FVIII-vWF
present in hemophilia A plasma (FVIII:C < 0.01 U/ml,
FVIII:C:Ag < 0.01 U/ml). Contrary to this, the
monoclonal antibodies present in these 23 ascitic fluids
did not react with plasma of a patient with severe
homogous von Willebrand's disease (FVIII:C 0.03 U/ml, FVIIIR:RCof < 0.001 U/ml, FVIIIIR:RCof < 0.06 U/ml). None of these 23 ascitic fluids showed binding to purified FVIIIIC:Ag or inhibited FVIII:C. The titer of these ascitic fluids (which contained between 1 and 1.5 mg IgG/ml ascites) with purified FVIII-vWF ranged from 10^6 to 10^10 (for nomenclature of these 23 cell lines, see legend to Table 1).

Two hybridoma ascitic fluids, CLB-CAg A and CLB-CAg F, selected for their ability to bind to purified FVIIIIC:Ag, bound to FVIII-vWF present in normal pooled plasma, but did not bind to FVIIIIC:Ag present in three hemophilia A plasmas. The titer of these two ascitic fluids (which contained between 1 and 1.5 mg IgG/ml ascites) with purified FVIII-vWF was 10^8. None of these 25 ascitic fluids showed binding to human fibrinogen, fibronectin, or IgG.

**Inhibition of FVIII:C**

An IgG preparation of monoclonal antibody CLB-CAg A (1 mg/ml) added to normal pooled plasma (1:1, 2 hr, at 37°C) inhibited FVIII:C completely. The Bethesda titer of this antibody was 133 U/mg IgG. No inhibition of FVIII:C was seen with monoclonal antibody CLB-CAg F.

**Inhibition of FVIIIR:RCof**

Of 25 ascitic fluids tested for their ability to inhibit FVIIIR:RCof, 2 ascites, CLB-RAg 34 and CLB-RAg 35, were able to inhibit the ristocetin-induced platelet aggregation completely. Complete inhibition of platelet aggregation was still observed when these ascitic fluids were diluted 4,000 times. No inhibition of collagen or adenosine diphosphate (ADP) induced platelet aggregation was seen with these monoclonal antibodies. The inhibition of ristocetin-induced aggregation of washed platelets by various amounts of purified CLB-RAg 35 IgG is shown in Fig. 1 as a dose–response curve. Complete inhibition of FVIIIR:RCof was observed at IgG concentrations greater than 1 μg/ml, whereas no inhibition was present at concentrations below 0.1 μg/ml. Similar results were seen with IgG of CLB-RAg 34.

When the incubation time of ascites and normal undiluted plasma was extended to 30 min instead of 10 min, 3 more monoclonal antibodies, CLB-RAg 7, CLB-RAg 38, and CLB-RAg 40, were able to inhibit FVIIIR:RCof, although not completely (Table 2). Ristocetin-induced aggregation was not inhibited by any other ascites, neither separately nor mixed altogether.

**Inhibition of Platelet Adherence to Subendothelium by Monoclonal Antibodies to FVIII-vWF**

The effect of 19 monoclonal antibodies to FVIII-vWF, which did not inhibit FVIII:C, on the adherence of platelets to subendothelium was investigated using an annular flow chamber and human umbilical arteries. In the absence of ascites or in the presence of control ascites (containing monoclonal antibodies against human granulocytes), platelet adherence was 65 × 10^6 platelets/sq cm. The monoclonal antibodies could be divided into 3 groups with respect to their influence on platelet adherence (Table 2). Group A monoclonal antibodies did not inhibit platelet adherence. Group B monoclonal antibodies partially inhibited platelet adherence to the level that is found when plasma of a patient with severe homozygous von Willebrand's disease is used. Group C monoclonal antibodies virtually abolished platelet adherence. The two
MONOCLONAL ANTIBODIES TO FVIII-vWF

Table 2. Inhibition of Platelet Adherence

<table>
<thead>
<tr>
<th>NP + Hybridoma</th>
<th>125I Platelet Adherence (Mean x 10^-3 sq cm)</th>
<th>Inhibition Platelet Adherence (%)</th>
<th>Inhibition FVIIIR:RCof (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP alone</td>
<td>65.2 (n = 9)</td>
<td>3.1</td>
<td>0</td>
</tr>
<tr>
<td>NP + CA</td>
<td>69.0 (n = 4)</td>
<td>3.8</td>
<td>0</td>
</tr>
<tr>
<td>vWD</td>
<td>30.5 (n = 4)</td>
<td>4.5</td>
<td>(53)</td>
</tr>
</tbody>
</table>

Group A

| NP + CLB-RAg 36 | 63.5 (n = 2) | 6.0 | 3 | 0 |
| NP + CLB-RAg 50 | 58.6 (n = 4) | 3.3 | 10 | 0 |
| NP + CLB-RAg 54 | 52.5 (n = 2) | 0.5 | 19 | 0 |
| NP + CLB-RAg 23 | 52.1 (n = 4) | 7.9 | 20 | 0 |
| NP + CLB-RAg 56 | 48.3 (n = 2) | 2.8 | 26 | 0 |

Group B

| NP + CLB-RAg 21 | 46.5 (n = 2) | 4.0 | 29 | 0 |
| NP + CLB-RAg 201 | 43.9 (n = 2) | 6.3 | 33 | 0 |
| NP + CLB-RAg 43 | 35.6 (n = 4) | 9.0 | 45 | 0 |
| NP + CLB-RAg 42 | 34.5 (n = 4) | 7.0 | 47 | 0 |
| NP + CLB-RAg 41 | 30.5 (n = 2) | 6.5 | 53 | 0 |
| NP + CLB-RAg 38 | 30.1 (n = 4) | 1.4 | 54 | 50* |
| NP + CLB-RAg 58 | 29.5 (n = 4) | 10.0 | 55 | 0 |
| NP + CLB-RAg 20 | 24.8 (n = 2) | 6.8 | 62 | 0 |
| NP + CLB-RAg 1 | 24.3 (n = 2) | 7.7 | 63 | 0 |
| NP + CLB-RAg 40 | 24.0 (n = 2) | 2.5 | 63 | 50* |
| NP + CLB-RAg 39 | 21.0 (n = 2) | 10.5 | 68 | 0 |
| NP + CLB-RAg 7 | 18.8 (n = 2) | 2.8 | 71 | 62* |

Group C

| NP + CLB-RAg 34 | 10.5 (n = 2) | 6.0 | 84 | 100 |
| NP + CLB-RAg 35 | 3.3 (n = 4) | 0.6 | 95 | 100 |

Inhibition of platelet adherence to human subendothelium by the various monoclonal antibodies. The standard deviation (SD) of the number of platelets that adhered with normal plasma alone was used to arbitrarily divide groups A, B, and C. Demarcation between groups A and B: number of adhered platelets in normal plasma alone minus two times SD; demarcation between groups B and C: number of adhered platelets in normal plasma alone minus five times SD. FVIIIR:RCof was measured in the plasmas that had been incubated with the ascitic fluids and was expressed as percentage inhibition of FVIIIR:RCof found in the plasma without incubation of ascitic fluid (FVIIIR:RCof and platelet adherence in this plasma set at 100%).

NP, normal plasma; vWD, von Willebrand’s disease plasma; SEM, standard error of mean; CA, control ascites; n, number of perfusions performed.

*Shorter incubation of normal plasma with these antibodies (10 min instead of 30 min) gave no inhibition of FVIIIR:RCof.

Antibodies that had this effect (CLB-RAg 34 and CLB-RAg 35) also blocked FVIIIR:RCof completely. As tested by immunodiffusion, these latter two antibodies did not form insoluble immune complexes, excluding the possibility that immunoprecipitation of FVIII-vWF was responsible for the observed inhibition of FVIIIR:RCof and platelet adherence to subendothelium.

Competition of Monoclonal Antibodies for Antigenic Epitopes

To investigate whether quantitative, rather than qualitative, effects of the monoclonal antibodies present in the various ascitic fluids were responsible for the degree of inhibition of platelet adherence to subendothelium, a monoclonal antibody in each group (Table 2; CLB-RAg 35, CLB-RAg 1, CLB-RAg 50) was radiolabeled and the competition for the same antigenic site with the various monoclonal antibodies was measured as described in Materials and Methods. The results of these experiments showed that monoclonal antibody CLB-RAg 34 completely abolished binding of 125I-labeled monoclonal antibody CLB-RAg 35 to FVIII-vWF (results not shown). This indicates that these two monoclonal antibodies are directed to the same (or closely related) epitope(s). No competition was seen between monoclonal antibody CLB-RAg 35 and the other monoclonal antibodies listed in Table 2 (including the monoclonal antibodies that partially inhibit the ristocetin-induced platelet aggregation—CLB-RAg 7, CLB-RAg 38, and CLB-RAg 40). Furthermore, it was observed that monoclonal antibodies CLB-RAg 1 and CLB-RAg 50, which are directed to different antigenic sites. None of the monoclonal antibodies, listed in Table 2, bound to the epitopes to which these latter two monoclonal antibodies were directed.

Gel Electrophoresis

After gel electrophoresis of cryoprecipitate, as well as of FVIII-vWF purified from factor VIII concentrate, and subsequent transfer to a nitrocellulose filter, FVIII-vWF could be visualized with radiolabeled antisera. Nonreduced samples in 1% agarose gel showed the multimeric composition of FVIII-vWF when the nitrocellulose filters were incubated with 125I-labeled IgG from a polyclonal rabbit anti-FVIII-vWF serum (Fig. 2). A similar band pattern resulted from incubation with all monoclonal antibodies (Fig. 2), except monoclonal antibodies CLB-RAg 7, CLB-RAg 21, CLB-RAg 22, CLB-CAg A, and CLB-CAg F, which did not show binding of antigen. When FVIII-vWF was reduced with mercaptoethanol in the presence of SDS at 37°C, only 7 of 21 antibodies tested bound to antigen after gel electrophoresis. All 7 monoclonal antibodies bound to antigen with an apparent molecular weight of 3.0 x 10^5. Three monoclonal antibodies bound also to antigen with an apparent molecular weight of 2.6 x 10^5. Only two antibodies (CLB-RAg 50 and CLB-RAg 52) visualized three bands with apparent molecular weights of 3.0 x 10^5, 2.6 x 10^5, and 2.0 x 10^5 (Fig. 3). Boiling the samples resulted in a further loss of antigenic determinants; only two antibodies, CLB-RAg 50 and CLB-RAg 52, visualized material with a molecular weight of 2.0 x 10^5 (Fig. 4).

Immunofluorescence

Indirect immunofluorescence staining of endothelial cells with ascitic fluids obtained from fusions 1 and 2...
Two monoclonal antibodies, selected in the initial screening of hybridomas for their binding to purified FVIII:C:Ag, bound to purified FVIII-vWF, to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in three hemophilia A plasmas. This indicates that these two antibodies are directed to antigenic determinants present on the factor VIII moiety of FVIII-vWF. One of these two antibodies inhibited FVIII:C completely.

Two of the 23 monoclonal antibodies directed against the von Willebrand factor moiety of FVIII-vWF described in this article (CLB-RAg 34 and CLB-RAg 35) distinguish themselves from previously described monoclonal antibodies\textsuperscript{22-24} by the following properties.

1. They inhibit FVIIIIR:RCof completely (Table I).
2. They bind to purified FVIII-vWF and to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in hemophilia A plasma.
3. They do not inhibit FVIII:C.
4. They distinguish themselves from previously described monoclonal antibodies.

**DISCUSSION**

A panel of 25 monoclonal antibodies directed against human FVIII-vWF was obtained using the hybridoma technique. Twenty-three monoclonal antibodies bound to purified FVIII-vWF, FVIII-vWF present in normal plasma, and FVIII-vWF present in hemophilia A plasma (Table I). None of these antibodies bound to purified FVIII:C:Ag or inhibited FVIII:C. These results indicate that these 23 monoclonal antibodies are directed against determinants present on the von Willebrand factor moiety of FVIII-vWF.

Two monoclonal antibodies, selected in the initial screening of hybridomas for their binding to purified FVIII:C:Ag, bound to purified FVIII-vWF, to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in three hemophilia A plasmas. This indicates that these two antibodies are directed to antigenic determinants present on the factor VIII moiety of FVIII-vWF. One of these two antibodies inhibited FVIII:C completely.

Two of the 23 monoclonal antibodies directed against the von Willebrand factor moiety of FVIII-vWF described in this article (CLB-RAg 34 and CLB-RAg 35) distinguish themselves from previously described monoclonal antibodies\textsuperscript{22-24} by the following properties.

1. They inhibit FVIIIIR:RCof completely (Table I).
2. They bind to purified FVIII-vWF and to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in hemophilia A plasma.
3. They do not inhibit FVIII:C.
4. They distinguish themselves from previously described monoclonal antibodies.

**DISCUSSION**

A panel of 25 monoclonal antibodies directed against human FVIII-vWF was obtained using the hybridoma technique. Twenty-three monoclonal antibodies bound to purified FVIII-vWF, FVIII-vWF present in normal plasma, and FVIII-vWF present in hemophilia A plasma (Table I). None of these antibodies bound to purified FVIII:C:Ag or inhibited FVIII:C. These results indicate that these 23 monoclonal antibodies are directed against determinants present on the von Willebrand factor moiety of FVIII-vWF.

Two monoclonal antibodies, selected in the initial screening of hybridomas for their binding to purified FVIII:C:Ag, bound to purified FVIII-vWF, to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in three hemophilia A plasmas. This indicates that these two antibodies are directed to antigenic determinants present on the factor VIII moiety of FVIII-vWF. One of these two antibodies inhibited FVIII:C completely.

Two of the 23 monoclonal antibodies directed against the von Willebrand factor moiety of FVIII-vWF described in this article (CLB-RAg 34 and CLB-RAg 35) distinguish themselves from previously described monoclonal antibodies\textsuperscript{22-24} by the following properties.

1. They inhibit FVIIIIR:RCof completely (Table I).
2. They bind to purified FVIII-vWF and to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in hemophilia A plasma.
3. They do not inhibit FVIII:C.
4. They distinguish themselves from previously described monoclonal antibodies.

**DISCUSSION**

A panel of 25 monoclonal antibodies directed against human FVIII-vWF was obtained using the hybridoma technique. Twenty-three monoclonal antibodies bound to purified FVIII-vWF, FVIII-vWF present in normal plasma, and FVIII-vWF present in hemophilia A plasma (Table I). None of these antibodies bound to purified FVIII:C:Ag or inhibited FVIII:C. These results indicate that these 23 monoclonal antibodies are directed against determinants present on the von Willebrand factor moiety of FVIII-vWF.

Two monoclonal antibodies, selected in the initial screening of hybridomas for their binding to purified FVIII:C:Ag, bound to purified FVIII-vWF, to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in three hemophilia A plasmas. This indicates that these two antibodies are directed to antigenic determinants present on the factor VIII moiety of FVIII-vWF. One of these two antibodies inhibited FVIII:C completely.

Two of the 23 monoclonal antibodies directed against the von Willebrand factor moiety of FVIII-vWF described in this article (CLB-RAg 34 and CLB-RAg 35) distinguish themselves from previously described monoclonal antibodies\textsuperscript{22-24} by the following properties.

1. They inhibit FVIIIIR:RCof completely (Table I).
2. They bind to purified FVIII-vWF and to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in hemophilia A plasma.
3. They do not inhibit FVIII:C.
4. They distinguish themselves from previously described monoclonal antibodies.

**DISCUSSION**

A panel of 25 monoclonal antibodies directed against human FVIII-vWF was obtained using the hybridoma technique. Twenty-three monoclonal antibodies bound to purified FVIII-vWF, FVIII-vWF present in normal plasma, and FVIII-vWF present in hemophilia A plasma (Table I). None of these antibodies bound to purified FVIII:C:Ag or inhibited FVIII:C. These results indicate that these 23 monoclonal antibodies are directed against determinants present on the von Willebrand factor moiety of FVIII-vWF.

Two monoclonal antibodies, selected in the initial screening of hybridomas for their binding to purified FVIII:C:Ag, bound to purified FVIII-vWF, to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in three hemophilia A plasmas. This indicates that these two antibodies are directed to antigenic determinants present on the factor VIII moiety of FVIII-vWF. One of these two antibodies inhibited FVIII:C completely.

Two of the 23 monoclonal antibodies directed against the von Willebrand factor moiety of FVIII-vWF described in this article (CLB-RAg 34 and CLB-RAg 35) distinguish themselves from previously described monoclonal antibodies\textsuperscript{22-24} by the following properties.

1. They inhibit FVIIIIR:RCof completely (Table I).
2. They bind to purified FVIII-vWF and to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in hemophilia A plasma.
3. They do not inhibit FVIII:C.
4. They distinguish themselves from previously described monoclonal antibodies.

**DISCUSSION**

A panel of 25 monoclonal antibodies directed against human FVIII-vWF was obtained using the hybridoma technique. Twenty-three monoclonal antibodies bound to purified FVIII-vWF, FVIII-vWF present in normal plasma, and FVIII-vWF present in hemophilia A plasma (Table I). None of these antibodies bound to purified FVIII:C:Ag or inhibited FVIII:C. These results indicate that these 23 monoclonal antibodies are directed against determinants present on the von Willebrand factor moiety of FVIII-vWF.

Two monoclonal antibodies, selected in the initial screening of hybridomas for their binding to purified FVIII:C:Ag, bound to purified FVIII-vWF, to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in three hemophilia A plasmas. This indicates that these two antibodies are directed to antigenic determinants present on the factor VIII moiety of FVIII-vWF. One of these two antibodies inhibited FVIII:C completely.

Two of the 23 monoclonal antibodies directed against the von Willebrand factor moiety of FVIII-vWF described in this article (CLB-RAg 34 and CLB-RAg 35) distinguish themselves from previously described monoclonal antibodies\textsuperscript{22-24} by the following properties.

1. They inhibit FVIIIIR:RCof completely (Table I).
2. They bind to purified FVIII-vWF and to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in hemophilia A plasma.
3. They do not inhibit FVIII:C.
4. They distinguish themselves from previously described monoclonal antibodies.

**DISCUSSION**

A panel of 25 monoclonal antibodies directed against human FVIII-vWF was obtained using the hybridoma technique. Twenty-three monoclonal antibodies bound to purified FVIII-vWF, FVIII-vWF present in normal plasma, and FVIII-vWF present in hemophilia A plasma (Table I). None of these antibodies bound to purified FVIII:C:Ag or inhibited FVIII:C. These results indicate that these 23 monoclonal antibodies are directed against determinants present on the von Willebrand factor moiety of FVIII-vWF.

Two monoclonal antibodies, selected in the initial screening of hybridomas for their binding to purified FVIII:C:Ag, bound to purified FVIII-vWF, to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in three hemophilia A plasmas. This indicates that these two antibodies are directed to antigenic determinants present on the factor VIII moiety of FVIII-vWF. One of these two antibodies inhibited FVIII:C completely.

Two of the 23 monoclonal antibodies directed against the von Willebrand factor moiety of FVIII-vWF described in this article (CLB-RAg 34 and CLB-RAg 35) distinguish themselves from previously described monoclonal antibodies\textsuperscript{22-24} by the following properties.

1. They inhibit FVIIIIR:RCof completely (Table I).
2. They bind to purified FVIII-vWF and to FVIII-vWF present in normal plasma, but not to FVIII-vWF present in hemophilia A plasma.
3. They do not inhibit FVIII:C.
4. They distinguish themselves from previously described monoclonal antibodies.
Based on these observations, it is concluded that ristocetin-induced binding of FVIII-vWF to platelets reflects, at least in part, a physiologic mechanism regulating the function of FVIII-vWF in primary hemostasis. This is corroborated by the observation that an absence of ristocetin-induced platelet aggregation is always associated with a prolonged bleeding time. On the other hand, a normal FVIII:RCoF with a prolonged bleeding time is observed, although only occasionally. This supports the concept that the bleeding time reflects not only platelet-vWF-platelet interaction, but also other processes (e.g., binding of plasma vWF to the subendothelium).

Monoclonal antibodies from group B that partially inhibited FVIII:RCoF activity (Table 2; CLB-RAg 38, CLB-RAg 40, CLB-RAg 7) presumably partially inhibit the interaction between platelets and FVIII-vWF, and this may explain the partial inhibition of platelet adherence by these antibodies.

Sakariassen et al. showed that plasma FVIII-vWF first binds to subendothelium and then mediates platelet adherence. A possible explanation for the inhibition of platelet adherence, seen with monoclonal antibodies from group B that do not inhibit FVIII:RCoF (Table 2), is that these antibodies inhibit binding of plasma FVIII-vWF to subendothelium. The platelet adherence observed in the presence of these antibodies can be explained by the interaction between platelets and vWF already present in the subendothelium. This view is supported by the observation that the platelet adherence to (normal) subendothelium, observed with von Willebrand’s disease plasma, is in the same range as the platelet adherence observed with normal plasma preincubated with these monoclonal antibodies.

Data from Turitto et al. indicate that indeed vessel wall vWF may contribute to platelet adherence. In addition, preliminary immunofluorescent studies with a limited number of our monoclonal antibodies indicated that these antibodies bind to vWF present in the subendothelium of the vessel wall (results not shown). These findings may explain the differences between platelet adherence in von Willebrand’s disease plasma and (normal) plasma incubated with antibodies from group C. In the latter case, these antibodies prevent both the contribution of plasma FVIII-vWF and vessel wall vWF to platelet adherence.

Monoclonal antibodies from group A (Table 2), which neither inhibit platelet adherence to subendothelium nor inhibit FVIII:RCoF, are probably directed to antigenic sites not primarily involved in the interaction between platelets and FVIII-vWF or to antigenic sites not involved in the binding of plasma FVIII-vWF to subendothelium. The gradual differences among the inhibition capacities of the monoclonal antibodies from groups A, B, and C might be
due to differences in affinity of these monoclonal antibodies toward one and the same epitope. However, this is unlikely, because the ascitic fluids were tested in excess, and the titer of the different ascitic fluids with FVIII-vWF did not correlate with the degree of inhibition of platelet adherence to subendothelium. Furthermore, from the displacement study with monoclonal antibody CLB-RAg 35 (group C), it appeared that this monoclonal antibody is directed against an epitope on FVIII-vWF that is not recognized by the monoclonal antibodies from groups A and B.

After gel electrophoresis of normal cryoprecipitate as well as of purified FVIII-vWF, the multimeric composition could be visualized by immunoblotting with monoclonal antibodies (Fig. 2). From the visualized multimers, it could be concluded that these antibodies (including CLB-RAg 35 and CLB-RAg 34) bind to epitopes present on all multimeric forms of FVIII-vWF. The fact that the antigenic site to which monoclonal antibodies CLB-RAg 35 and CLB-RAg 34 are directed is also present on the low molecular weight multimers that do not express FVIIIR:RCof activity suggests that small multimers of FVIII-vWF possess potential FVIIIR:RCof and von Willebrand factor activities that are only fully manifested following formation of large FVIII-vWF multimers. That possibly a limited region of the molecule is functionally active in this respect is corroborated by the findings of Martin et al., who showed residual FVIIIR:RCof activity in a relatively small fragment (molecular weight 116,000) of FVIII-vWF.

The monoclonal antibodies CLB-RAg 7, CLB-RAg 21, CLB-RAg 22, CLB-CAg A, and CLB-CAg F, which were not able to bind to FVIII-vWF after gel electrophoresis, were apparently directed to native FVIII-vWF only. An alternative explanation is that these monoclonal antibodies bind to a hydrophobic binding site on the factor VIII molecule that is blocked by SDS.

The majority of the monoclonal antibodies produced bind only to unmodified FVIII-vWF. Partial or complete denaturation, accomplished by incubation with mercaptoethanol and SDS at different temperatures, leads to a loss of antibody binding sites (Figs. 3 and 4). This suggests that these antibodies (including CLB-RAg 34 and CLB-RAg 35) are directed against conformational determinants. Only two monoclonal antibodies, CLB-RAg 50 and CLB-RAg 52, bound to the subunit of 2.0 x 106 of FVIII-vWF (Fig. 4). These two antibodies are most likely directed to antigenic determinants related to the primary sequence of FVIII-vWF rather than to its conformational structure.

In this context, it is interesting to note that both endothelial cells and platelets show binding of monoclonal antibodies that do not react with denatured FVIII-vWF. This indicates that, in these cells, the antigen, at least in part, is present in a stable conformation.

REFERENCES

5. Tschopp T, Weiss JH, Baumgartner HR: Decreased adhesion of platelets to subendothelium in Von Willebrand’s disease. J Lab Clin Med 83:296, 1974
MONOCLONAL ANTIBODIES TO FVIII-vWF


24. Ogata K, Saito H, Ratnoff OD: The relationship of the properties of antihemophilic factor (factor VIII) that support ristocetin-induced platelet agglutination (factor VIIIIR:Rc) and platelet retention by glass beads as demonstrated by a monoclonal antibody. Blood 61:27, 1983


Characterization of 25 monoclonal antibodies to factor VIII-von Willebrand factor: relationship between ristocetin-induced platelet aggregation and platelet adherence to subendothelium

HV Stel, KS Sakariassen, BJ Scholte, EC Veerman, TH van der Kwast, PG de Groot, JJ Sixma and JA van Mourik