Direct Radioimmune Detection in Human Plasma of the Association Between Factor VIII Procoagulant Protein and von Willebrand Factor, and the Interaction of von Willebrand Factor-Bound Procoagulant VIII With Platelets

By Joel L. Moake, Mark J. Weinstein, Joseph H. Troll, Leslie E. Chute, and Noreen M. Colannino

The predominant procoagulant factor VIII (VIII:C) form in normal human plasma contains various combinations of anticoagulants and serine/cysteine protease inhibitors is a protein with mol wt 2.6 ± 0.2 x 10^6. This protein can be detected by 125I-anti-VIII:C Fab binding and gel electrophoresis in the presence and absence of sodium dodecylsulfate (SDS) and is distinct from the subunit of factor VIII/von Willebrand factor (VIII:vWF) multimers. No larger VIII:C form is present in plasma from patients with severe congenital deficiencies of each of the coagulation factors, other than VIII:C. The mol wt ~2.6 x 10^6 VIII:C form is, therefore, likely to be the in vivo procoagulant form of VIII:C, rather than a partially proteolyzed, partially activated derivative of a larger precursor. About 60% of this procoagulant mol wt ~2.6 x 10^6 VIII:C form in plasma is present in noncovalent complexes with larger VIII:vWF multimers, which attach reversibly to platelet surfaces in the presence of ristocetin. This VIII:vWF-bound protein of mol wt ~2.6 x 10^6 may be the plasma procoagulant form of VIII:C which, after proteolytic activation, accelerates the IXa-mediated cleavage and activation of X postulated to occur on platelet surfaces.

EVIDENCE HAS ACCUMULATED that the human factor VIII complex is composed of two different proteins under distinct genetic control and linked by noncovalent bonds. The smaller of the two VIII complex components is procoagulant factor VIII (VIII:C) and the larger is von Willebrand factor (VIII:vWF), a multimer of disulfide-linked 2.3 x 10^6 dalton subunits. Human VIII:vWF augments platelet-subendothelial interactions and adsorbs to (and agglutinates) human platelets in vitro if platelet negative surface charges are neutralized partially by the cationic glycoprotein antibiotic, ristocetin.

The plasma form of procoagulant VIII:C protein is controversial, with size estimates ranging from 0.79–2.8 x 10^6 daltons for the human molecule to 0.85–3.0 x 10^6 for bovine VIII:C. The precise relationship between procoagulant VIII:C and VIII:vWF multimers in plasma has also been elusive. Difficulties in isolating and characterizing the procoagulant form of VIII:C, and in defining the VIII:vWF multimer forms to which procoagulant VIII:C molecules attach, reflect the small quantity of VIII:C protein (~50 ng/ml) in normal plasma. Furthermore, VIII:C is susceptible to cleavage by trace quantities of Xa, thrombin, plasmin, and activated protein C. It is not known if VIII:C can be proteolyzed by activated contact coagulation factors or by other serine and cysteine proteases.

This study was designed to determine conclusively the circulating plasma form of procoagulant VIII:C, and the extent of association between this molecule and VIII:vWF multimers. Immunologic techniques were used to study the relationship directly in: (1) normal human platelet-poor plasma (PPP) prepared from blood collected under conditions that prevent ex vivo activation—and inhibit rapidly any ex vivo activi-ty—of serine and cysteine proteases; and (2) plasmas from patients severely deficient in each one of the coagulation factors which, after in vivo activation, might proteolyze or otherwise alter the circulating procoagulant form of VIII:C.

MATERIALS AND METHODS

Preparation of Plasma and Platelets

Blood from normal donors was collected using a tourniquet and a two-syringe technique. The first 5 ml of blood was discarded, and the remainder was collected into plastic syringes containing anticoagulants and proteolytic inhibitors in various combinations (Table 1) including: 0.38% sodium citrate alone (final concentration) or acid-citrate-dextrose (ACD), (p-amidinophenyl) methanesulfonyl fluoride (p-APMSF, kindly provided by Dr. D. H. Bing), disopropylfluorophosphate (DFP), hexadimethrine bromide (Aldrich Chemical Co., Milwaukee, WI), HgCl₂, iodoacetamide, hirudin (Sigma Chemical Co., St. Louis, MO), and 14 USP U/ml heparin (from porcine intestinal mucosa, Abbott Laboratories, Chicago, IL). Thrombin, Xa, plasmin, and probably others among the coagulation serine proteases are rapidly, irreversibly, and completely inhibited by the benzamidine derivative, p-APMSF, which covalently sulfonlates active site serine residues. Coagulation serine proteases are also inactivated by DFP, and rapidly by antithrombin-III in the presence of heparin. Cysteine proteases, including a factor-X-activating enzyme, are reversibly inhibited by HgCl₂ and irreversibly inhibited by iodoacetamide. Hirudin inhibits thrombin,
and hexadimethrine bromide inhibits the surface activation of both XII and XIIa.15,16

The blood was transferred to polypropylene tubes, and platelet-poor plasma (PPP) was obtained by centrifugation at 800 g for 15 min at 5°C. PPP was also prepared from citrated blood in glass tubes containing no inhibitors. Citrated PPP from patients with severe deficiencies of single coagulation factors (XII, prekallikrein, high molecular weight kininogen, XI, IX, X, VII, V, prothrombin, fibrinogen, or XIII) was obtained from Boston area patients and from George King Bio-Medical, Inc. (Overland Park, KS). No additional inhibitors were added. After preparation, PPP and other experimental samples were frozen at −80°C for subsequent analysis. Formalinized platelets were prepared by a modification of established procedures.19 Before each experiment, the formalinized platelets were sedimented and resuspended in 0.028 M sodium barbital−0.125 M NaCl, pH 7.4 (veronal-buffered saline, VBS).

Assays of VIII Complex Components

VIII:C activity was measured in a standard one-state activated partial thromboplastin time test system using severe hemophilia A plasma as substrate.21 VIII:C antigen (VIII:CAg) was quantified by fluid phase immunoradiometric assay using human 125I-anti-VIII:C Fab,22 and VIII:vWF antigen (VIII:vWF Ag) by solid phase immunoradiometric assay using rabbit anti-human VIII:vWF (Dako Antibodies, Accurate Chemical and Scientific Corp., Westbury, NY) and rabbit 125I-anti-human VIII:vWF.13

Preparation of 125I-Anti-VIII:C Fab

Anti-VIII:C IgG was purified from a hemophilia A patient with high titer VIII:C inhibitor, and Fab fragments were produced with papain. These Fab fragments were separated and radiiodinated, and specific 125I-anti-VIII:C Fab fragments were isolated as described previously.6 The 125I-anti-VIII:C Fab had a specific activity of 20,000 NIH U/mg and did not react with VIII:vWF Ag (as shown in Results).

Gel Electrophoretic Analysis

(1) SDS-3% Polyacrylamide/0.5% Agarose

Samples (30 μl) of 1:8 PPP in VBS were incubated with 125I-anti-VIII:C Fab (33 μl, 9,000 cpm), containing 5 mM DFP, 95 NIH U/ml hirudin, 367 kallikrein inhibitor U/ml aprotinin (Trasylol, FBA Pharmaceuticals, New York, NY), 2 μM p-APMSF, and 3.2% polyethylene glycol (average mol wt 4,000) for 1.5 hr at 37°C and 30 min at 0°C, and then rewarmed at 23°C for 15 min. Thirty-three microliters of this mixture were added to 33 μl of a 0.02 M Tris-HCl–2 mM EDTA–8 M urea–2% sodium dodecylsulfate (SDS) solution, pH 8.0. After incubation for 20 min at 37°C, a 30-μl portion of each Tris-EDTA-urea-SDS solubilized sample was applied to a well in a 3% polyacrylamide/0.5% agarose gel slab (ME agarose, Marine Colloids Division, FMC Corp., Rockland, ME) for 4-7 days at −70°C.

Table 1. Combinations of Anticoagulants and Inhibitors

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<tr>
<th>Anticoagulant</th>
<th>Serine Protease Inhibitor(s)</th>
<th>Inhibitor of XII Activation/Activity</th>
<th>Cysteine Protease Inhibitor</th>
<th>Thrombin Inhibitor</th>
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<td>Citrate</td>
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<td>Citrate</td>
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<td>Hexadimethrine Br†</td>
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<td>Heparin + heparin</td>
<td>p-APMSF + DFP</td>
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*1 p-Argininophenylmethanesulfonyl fluoride, 1 μM (final concentration).
†0.1 mM.
§1 mM.
| ACD + heparin | p-APMSF + DFP                | lodoacetamide               | Hirudin

*1 50 μg/ml or 200 μg/ml.
†0.1 M.
§1 M.
| ACD p-APMSF + Hexadimethrine Br | lodoacetamide               | Hirudin
| Heparin p-APMSF + DFP | lodoacetamide               | Hirudin

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samples in the Tris-EDTA-urea-SDS solution for times ranging from 1 min to 1 hr before electrophoresis did not alter the relative intensity of the VIII:CAg forms. This indicates that the largest VIII:CAg detected (mol wt 2.6 ± 0.2 x 10^4) was not converted by experimental conditions to smaller VIII:CAg forms. The most intense banding patterns, however, were obtained at incubation times between 5 and 20 min, and 20-min incubation periods were used in all experiments.

The apparent molecular weight values for the antigen–antibody complexes were determined by interpolation on a straight line derived by plotting migration distance versus the logarithm of molecular weight values obtained for a2-macroglobulin, ferritin, fibronectin, fibrinogen, IgG, thyroglobulin, albumin, and phospholipase b in Coomassie-blue-stained gel lanes. An approximate molecular weight for each VIII:CAg form in the antigen–antibody complexes was obtained indirectly by subtracting a molecular weight value of 5 x 10^4 for the single 125I-anti-VIII:C Fab fragment that has been shown to be present in each VIII:CAg + 125I-anti-VIII:C Fab complex.

(2) 0.3%/2% Agarose (Without SDS)

For each slab gel, 2% agarose (1.5 x 60 x 180 mm) was poured in 0.08 M Tris-0.024 M Tricine running buffer, pH 8.6. A 0.3% agarose gel (1.5 x 30 x 180 mm) was poured adjacent to the 2% slab, with the well-forming comb (BioRad, Richmond, CA) placed 1.5 cm from the 2% gel. Dilutions of PPP samples were incubated with 125I-anti-VIII:C Fab exactly as in (1) above, and 30 μl of the mixtures were applied to the wells. Electrophoresis was in the horizontal position for 20 min at 50 V, 15 min at 100 V, and 4 hr at 150 V. When the bromphenol blue tracking dye reached the 1.5 cm from the 2% gel. Dilutions of PPP samples were incubated with 125I-anti-VIII:C Fab exactly as in (1) above, and 30 μl of the mixtures were applied to the wells. Electrophoresis was in the horizontal position for 20 min at 50 V, 15 min at 100 V, and 4 hr at 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the 150 V. When the bromphenol blue tracking dye reached the

(3) SDS-1 % Agarose

This autoradiographic technique has been described. Rabbit 125I-anti-human VIII:vWF IgG (1 x 10^5 cpm/ml) was used for gel overlay.

(4) Two-Dimensional Crossed-Immunoelectrophoresis (Without SDS)

Samples (50 μl) of normal PPP were incubated as in (1) above with 30 μl of the 125I-anti-VIII:C Fab solution (15,000 cpm). Aliquots (30 μl) were applied to a horizontal 0.4% agarose slab gel (1.5 x 100 x 180 mm) (IsoGel, Marine Colloids) in barbital-acetate buffer (0.048 M sodium barbital-0.08 M sodium acetate, pH 8.6), and electrophoresed at 50 V for 20 min, 100 V for 15 min, and 150 V for 150 min. The first-dimensional gel lane was removed and abutted to a 1.5 x 40 x 60 mm 0.5% agarose gel in barbital-acetate buffer (0.016 M sodium barbital-0.027 M sodium acetate, pH 8.6) containing a 1/50 dilution of rabbit anti-human VIII:vWF (Calbiochem-Behring, LaJolla, CA). Electrophoresis in the second dimension was at 40 V for 16 hr using the first-dimensional running buffer. Gels were washed for 18 hr in 0.15 M NaCl (two changes), and then for 2 hr in water (two changes) with alternate washing and pressing. They were stained with 0.27% Coomassie blue, and autoradiograms were developed for 1–2 wk as in (1) above.

Attachment of VIII Complex Components to Platelets in the Presence of Ristocetin

One volume of citrated PPP was mixed with 3 volumes of a formalinized platelet suspension in VBS (or VBS alone for zero platelet controls) in 1.5 ml polypropylene microcentrifuge tubes. Either ristocetin (BioData, Horsham, PA) in VBS (1.25 mg/ml) or VBS alone for zero ristocetin controls was added, and the tubes were incubated with rocking for 15–20 min at 37°C. Platelet agglutination occurred in the presence, but not in the absence, of ristocetin. Final incubation volumes, which ranged from 450 to 555 μl, were identical within each experimental set. The tubes were centrifuged at 8,000 g for 15 sec. Exactly 95% of supernatant volume was then removed from each tube using an adjustable volumetric micropette. Sedimented platelets were resuspended in VBS (without ristocetin) either at 50% or 100% of original volume, incubated at 23°C for 20–30 min with rocking, centrifuged again at 8,000 g for 15 sec, and the wash solution removed. Supernatant and wash samples were tested immediately for VIII:C activity. The other analyses (immunoradiometric and gel electrophoretic studies) were either done immediately, or subsequently on samples that had been quickly frozen at −70°C. In these experiments (Figs. 4A and 5A), "percent attachment to platelets" was the quantity of VIII:C activity, VIII:CAg or VIII:vWF Ag that disappeared from plasma supernatant samples in the presence of formalinized platelets and ristocetin, relative to the concentration of each VIII complex component in control plasma supernatant samples that contained either platelets and no ristocetin (100% values for Fig. 4A) or ristocetin and no platelets (100% values for Fig. 5A). The "percent recovered from platelets" (Figs. 4B and 5B) was the quantity of each VIII complex component recovered in platelet wash solutions relative to the control plasma supernatant samples (100% values). Nonspecific attachment of VIII complex components to the walls of sample tubes was <10% of the total in plasma supernatant experiments and <5% in wash solution studies. These background values were subtracted prior to the calculation of "percent attachment to platelets" and "percent recovered from platelets."

RESULTS

The forms of procoagulant VIII:C and the relationship between VIII:C and VIII:vWF in normal plasma were studied. Blood was collected and PPP was prepared in various combinations of anticoagulants (citrate, acid citrate dextrose (ACD), or heparin) and inhibitors of activated coagulation factors and other proteolytic enzymes (hexadimethrine bromide, p-APMSF, DFP, hirudin, and HgCl2 or iodoacetamide). These PPP samples, as well as plasma samples from patients with severe congenital deficiencies of factor XII, prekallikrein, high molecular weight kininogen, XI, IX, X, VII, V, prothrombin, fibrinogen, or factor XIII, were first incubated with 125I-anti-VIII:C Fab and analyzed by SDS-polyacrylamide/agarose electrophoresis and autoradiography.

The patterns obtained in normal plasma samples containing all combinations of anticoagulants and proteolytic inhibitors, in citrated PPP prepared in glass without proteolytic inhibitors, and in the coagulation factor-deficient plasmas were all identical to the gels.
shown in Fig. 1. In all of these plasmas, the largest and quantitatively predominant VIII:C Ag + 125I-anti-VIII:C Fab complex was a broad band with upper and lower molecular weight limits of ~3.3 x 10^5 and 2.9 x 10^5, respectively (Fig. 1A). Molecular weight values for the upper and lower limit of this predominant VIII:C Ag band were then calculated to be ~2.8 and 2.4 x 10^5, respectively, by subtracting the 0.5 x 10^5 mol wt of 125I-anti-VIII:C Fab from 2.5, 1.85, and 1.7, respectively.

With our techniques, other VIII:C Ag forms might have escaped detection if they had remained associated with VIII:vWF multimers in SDS and, therefore, failed to penetrate into the polyacrylamide/agarose gels. If this had occurred, we would have detected larger VIII:vWF forms that had minimal electrophoretic mobility in 2% agarose after the autoradiogram was exposed for 20 hr (Fig. 2B). When exposure was prolonged to 50 hr, more rapidly migrating VIII:C Ag positions appeared that were probably associated with smaller VIII:vWF forms.

Figure 3 shows that the migration of plasma VIII:C Ag (detected by 125I-anti-VIII:C Fab) on two-dimensional immunoelectrophoresis (without SDS) is exactly concordant with plasma VIII:vWF Ag and that radioactivity is most obvious in areas where the relatively slower migrating, larger VIII:vWF Ag forms have been immunoprecipitated. Any VIII:C Ag bound to 125I-anti-VIII:C Fab, but not in complexes with VIII:vWF, migrates rapidly through the gel in the first dimension and escapes detection by this technique.

The results presented in Figs. 1B, 2 and 3 imply that VIII:C Ag is associated, to a considerable extent, with larger, less penetrating, relatively slowly migrating VIII:vWF Ag forms. This may be because relatively larger VIII:vWF multimers comprise the greater proportion of plasma VIII:vWF forms. The techniques used in our studies do not permit stoichiometric relationships between VIII:C Ag and VIII:vWF multimer forms to be ascertained precisely.

Immunoradiometric assays were used in conjunction with ristocetin-induced platelet agglutination and gel electrophoretic procedures. As ristocetin concentrations were increased above 0.7 mg/ml and formalinized platelets above 5 x 10^5/µl, there was progressively greater, and concomitant, attachment to platelets of VIII:vWF Ag and VIII:C Ag (with VIII:C activity)
Fig. 2. Association of VIII:CAg and VIII:vWF in normal plasma. (A) Normal citrated platelet-poor plasma (PPP) was incubated with 
\(^{125}\)I-anti-VIII:C Fab, and an aliquot was electrophoresed without SDS into a 0.3%/2% agarose gel slab. Arrow indicates the position of the 
predominant VIII:CAg form. (B and C) Normal citrated PPP was electrophoresed without SDS directly into another lane of the 0.3%/2% 
agarose gel slab, overlaid with rabbit \(^{125}\)I-anti-VIII:vWF IgG, and autoradiograms were obtained after exposure of film for 20 hr (B) or 50 hr 
(C). Positive pole is at the bottom. Relative gel positions of larger and smaller forms of VIII:vWF can be seen by comparing B (large plasma 
forms detected at 0.3%/2% gel interface) with C (all plasma forms are detected after longer film exposure). Identical patterns to those in B 
and C were obtained when PPP was incubated before electrophoresis with nonradioactive anti-VIII:C Fab.

(Figs. 4A and 5A). A considerable portion of this platelet-associated VIII:vWF Ag and VIII:CAg (with some persistent VIII:C activity) was recovered when 
the platelets were resuspended in VBS in order to dilute ristocetin to a concentration below that necessary to sustain VIII:vWF multimer attachment to 
platelets (Figs. 4B and 5B).

Following ristocetin-induced attachment of VIII complex components to platelets, subsequent recovery of VIII:C, VIII:CAg, and VIII:vWF diminished as 
initial ristocetin concentrations exceeded 1.5 mg/ml, or as initial platelet counts exceeded 4.5 \( \times 10^5/\mu l \). The former observation was the result of persistence in the 
wash solution of relatively more ristocetin with the
platelets and residual plasma. The latter finding probably reflected a less thorough dispersal and washing of the very large platelet clumps formed at high initial platelet counts in the presence of ristocetin and plasma.

Experiments were then done using normal citrated PPP, 1.2 mg/ml ristocetin, and 3–6 × 10⁵ formalinized platelets/μl. Results of one of these studies using 6 × 10⁵ platelets/μl will be described. (Identical results were obtained with 3 × 10⁵ platelets/μl.) On SDS-polyacrylamide/agarose gels (Fig. 6A) and densitometric scanning (Fig. 6B), it was found that about 60% of the total VIII:CaG in normal PPP, and about 60% of the predominant plasma mol wt ~2.6 × 10⁵ VIII:CaG form, was associated with the VIII:vWF multimers that preferentially attached to formalinized platelets in the presence of ristocetin (and caused platelet agglutination). These findings correlate well with the 60%–70% of total plasma VIII:CaG determined by immunoradiometric assay to bind to platelets in the presence of 1.2 mg/ml ristocetin (Figs. 4A and 5A). The VIII:CaG associated with these platelet-bound VIII:vWF multimers also included small quantities of the 2.0, 1.35, and 1.2 × 10⁵ mol wt forms, as well as the predominant mol wt ~2.6 × 10⁵ VIII:CaG (Fig. 6A and B).

The VIII:vWF multimers that attached to platelets in the presence of ristocetin were the relatively large
Fig. 5. Effect of platelet numbers on the attachment of plasma VIII complex components to platelets. Fresh normal citrated platelet-poor plasma diluted 1:3 with veronal-buffered saline was incubated for 20 min at 37°C with 1.2 mg/ml ristocetin and varying numbers of formalinized platelets. Quantification of supernatant (A) and eluate (B) VIII:vWF Ag, VIII:C Ag, and VIII:C activity was as in the legend to Fig. 2.
Fig. 6. Attachment of plasma VIII:C antigen to platelets. Normal citrated platelet-poor plasma diluted 1:2 with veronal-buffered saline was incubated for 20 min at 37°C with $6 \times 10^5$/μl formalinized platelets in the absence (1) or presence (2) of 1.2 mg/ml ristocetin. Platelets were sedimented by centrifugation, and platelet eluates obtained. (3) Platelet eluate from 1; (4) platelet eluate from 2. Supernatant and eluate samples were incubated with $^{125}$I-anti-VIII:C Fab, electrophoresed in SDS into 3% polyacrylamide/0.5% agarose, and the autoradiogram was prepared (A). Positive pole is at the bottom. The arrow indicates position of the predominant plasma VIII:CAg form identified by reaction with $^{125}$I-anti-VIII:C Fab. (B) Densitogram made by scanning the autoradiogram shown in A.

VIII:CAg that was not associated with the largest VIII:vWF multimers did not become attached to platelets in the presence or absence of ristocetin. This is the VIII:CAg that penetrated a considerable distance beyond the 0.3%/2% agarose interface into the 2% agarose in gel lanes 2 and 3 of Fig. 8.

No radioactivity was associated with VIII:vWF in the plasma of severe hemophilia A patients with normal levels of VIII:vWF (lane 1 of Fig. 8). This result confirms that there was no antigenic cross-reactivity between VIII:CAg forms and VIII:vWF, using radioactive Fab fragments made from one particular human anti-VIII:C IgG antibody that neutralizes VIII:C activity.

DISCUSSION

In normal human plasmas prepared in the presence of rapidly acting inhibitors of XII activation and serine/cysteine proteases, as well as in individual plasmas deficient in each one of the coagulation factors that might cleave or alter procoagulant VIII:C in vivo, the largest and quantitatively predominant form of VIII:CAg is a protein with an estimated mol wt of $2.6 \pm 0.2 \times 10^5$. Even in fresh citrated normal PPP prepared in glass without added inhibitors (containing XIIa, kallikrein, and Xla, which do not require Ca$^{2+}$ for activation$^{25}$), the $2.6 \times 10^5$ mol wt VIII:CAg form is predominant. This VIII:CAg form in fresh plasma is probably not, therefore, derived from a larger precursor protein as a result of in vivo or ex vivo proteolytic cleavage by XIIa, kallikrein, Xla, IXa, Xa, VIIa, thrombin, plasmin, activated protein C (for which VIII:C is a preferred substrate$^{12}$) or other serine protease, or a cysteine protease, that might degrade VIII:C either directly or indirectly (i.e., via X activation to Xa$^{21}$). The predominant mol wt $2.6 \times 10^5$ VIII:CAg, which can be cleaved and activated in vitro...
Fig. 7. Attachment of plasma VIII:vWF to platelets. Normal citrated platelet-poor plasma diluted 1:2 with veronal-buffered saline was incubated for 20 min at 37°C with 6 x 10⁵/μl formalinized platelets in the absence (1) or presence (2) of 1.2 mg/ml ristocetin. Platelets were sedimented by centrifugation, and platelet eluates obtained. (3) Platelet eluate from 1; (4) platelet eluate from 2. Supernatant and eluate samples were electrophoresed in SDS-1% agarose, overlaid with rabbit 125I-anti-VIII:vWF IgG, and autoradiograms were prepared.

Fig. 8. Attachment of VIII:vWF-bound VIII:C antigen to platelets. Normal citrated platelet-poor plasma diluted 1:2 with veronal-buffered saline was incubated for 20 min at 37°C with 6 x 10⁵/μl formalinized platelets in the absence (2) or presence (3) of 1.2 mg/ml ristocetin. Platelets were sedimented by centrifugation, and platelet eluates obtained. (4) Platelet eluate of 2; (5) platelet eluate of 3. (1) Undiluted plasma from patient with severe hemophilia A. Supernatants and platelet eluates, as well as hemophilia A plasma, were incubated with 125I-anti-VIII:C Fab, electrophoresed into 0.3% or 2% agarose without SDS, and autoradiograms were prepared. Positive pole is at the bottom. The arrow indicates the position of the predominant plasma VIII:C Ag-form identified by reaction with 125I-anti-VIII:C Fab.
VIII:CAg/vWF complexes with VIII:vWF multimers of relatively lower molecular weight (the intermediate mobility VIII:CAg form in 2% agarose gel lanes of Fig. 8).

Platelets may provide effective surfaces for the optimal interaction between VIII:C and platelet-bound factor IXa. The result, after protease activation of procoagulant VIII:C,12,34 is believed to be accelerated proteolysis and activation of platelet-bound factor X35 by VIII:C-IXα complexes. The ~60% of plasma mol wt ~2.6 × 10^5 procoagulant VIII:CAg that gains access to platelets in the ristocetin model system is present in VIII:CAg/vWF complexes which contain the relatively large plasma VIII:vWF multimers. It is possible that relatively large VIII complexes bind preferentially to platelets in vivo, perhaps following stimulation of platelets by thrombin (or other agent).36,37 If this is so, then the mol wt ~2.6 × 10^5 VIII:CAg in these large VIII complexes may be the procoagulant VIII:CAg form that participates—after activation by thrombin, Xa, or some other protease—in the acceleration of IXα-mediated cleavage and activation of X on platelet surfaces.

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REFERENCES

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