Interaction of Platelet Membrane Receptors with von Willebrand Factor, Ristocetin, and the Fc Region of Immunoglobulin G

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ABSTRACT The agglutination of human platelets by ristocetin and von Willebrand factor was inhibited by aggregated immunoglobulin (Ig)G and by Fc fragments of IgG, but not by Fab, $F(ab')_2$ or pFc fragments of IgG. Because this inhibition occurred with formalin-fixed platelets as well as with normal platelets, a generalized aggregation of fluid membrane components by Fc fragments was not responsible for this inhibition of ristocetin and von Willebrand factor-induced agglutination. Reciprocal inhibition of platelet Fc receptors was produced by prior incubation of platelets with von Willebrand factor and ristocetin. Sucrose density gradient ultracentrifugation studies demonstrated that aggregated IgG did not form fluid-phase complexes with von Willebrand factor and ristocetin. Furthermore, passage of von Willebrand factor and ristocetin through a column of immobilized heat-aggregated IgG did not alter platelet agglutinating activity which indicates that aggregated IgG did not inactivate von Willebrand factor or ristocetin. Thus, it was likely that the IgG-mediated interference with platelet agglutination by ristocetin and von Willebrand factor did not occur in the fluid phase but at the platelet surface. These studies suggest that the platelet membrane Fc receptor may be either a part of, or sterically related to, the membrane glycoprotein I complex that interacts with von Willebrand factor, and that occupation of one of these surface components blocks the availability of the other.

Part of this work has been previously published in abstract form: 1977. *Clin. Res.* **25**: 344A. (Abstr.)

Dr. Moore is a recipient of the Mary Putnam Jacobi Fellowship of the Women's Medical Association of the City of New York. Dr. Ross is an Established Investigator of the American Heart Association and a Career Scientist of the Irma T. Hirschl Charitable Trust. His present address is: Division of Immunology and Rheumatology, University of North Carolina Medical School, Chapel Hill, N. C. 27514.

Received 12 January 1978 and in revised form 21 July 1978.

INTRODUCTION

Normal platelets in platelet-rich plasma, or formalin-fixed platelets in the presence of normal plasma will agglutinate in response to the antibiotic ristocetin. The plasma factor that is required for this agglutination, von Willebrand factor, is part of the Factor VIII complex and is absent or diminished in patients with the inherited bleeding disorder, von Willebrand's disease (1). Recent studies of the exact mechanism by which ristocetin supports von Willebrand factor-dependent platelet agglutination suggest that ristocetin and von Willebrand factor bind to the platelet and cause a reduction in net surface charge. This reduction in charge allows the large von Willebrand factor molecule to serve as an effective bridge between platelets, and agglutination occurs (2, 3). A specific platelet surface membrane glycoprotein of mol wt 155,000, glycoprotein I, appears to be the mediator for the ristocetin-induced von Willebrand factor-dependent platelet reaction (4–6).

Soluble immune complexes and aggregated immunoglobulin (Ig)G also interact with washed platelets, causing aggregation and the release of intracellular serotonin (7, 8). The $F(ab')_2$ fragments of IgG do not cause platelet aggregation, and preincubation of platelets with IgG Fc fragments blocks the aggregation induced by aggregated IgG (7, 9). Platelet aggregation, under these circumstances, does not require complement components and appears to be initiated by the direct interaction of aggregated IgG or immune complexes with a platelet Fc receptor (7, 10). In contrast to washed platelets, platelets in plasma do not aggregate or release serotonin in response to aggregated IgG. However, aggregated IgG does bind to platelets in plasma as well as to washed platelets or formalin-fixed platelets (11, 12).

In this present study, it was observed that aggregated IgG and, specifically, purified Fc fragments of IgG,

blocked the agglutination response of platelets to von Willebrand factor and ristocetin. In addition, von Willebrand factor in the presence of ristocetin blocked the uptake of soluble immune complexes by the platelet Fc receptor. Further experiments were then performed to explore the nature of the interactions which involve von Willebrand factor, ristocetin, and the Fc region of IgG.

METHODS

Platelet-rich plasma (PRP),[1] *platelet-poor plasma (PPP), and formalin-fixed washed platelets (FWP).* Blood drawn from healthy volunteers was mixed with 0.1 vol of 3.2% sodium citrate in polypropylene tubes and centrifuged at 225 *g* for 10 min at room temperature to sediment leukocytes and erythrocytes. Platelet counts were performed with a Coulter Counter, model ZBI (Coulter Electronics Inc., Hialeah, Fla.), and the PRP was diluted with PPP to a platelet count of 200,000/μl. PPP was prepared from PRP by centrifugation at 1,100 *g* for 20 min at room temperature. FWP were prepared from whole blood by the method of Grant et al. (13) and stored at 4 °C. For use in the microtiter aggregation assay, the FWP were resuspended in fresh PPP to a final concentration of 200,000/μl.

Antisera. Rabbit antisera to human albumin, ceruloplasmin, fibrinogen, IgG, and α_2-macroglobulin were obtained from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. Antisera to IgA, IgM, and IgD were prepared by immunization of rabbits with myeloma proteins purified by starch block electrophoresis and Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N. J.) column chromatography. The antisera to IgD, IgM, and IgA were absorbed with agarose-conjugated plasma proteins and shown to be specific for δ-, μ-, and α-heavy chains. Antiserum to C3 was produced by immunization of rabbits with purified human C3 (14). Antiserum to the hapten fluorescein isothiocyanate (FITC, Calbiochem, San Diego, Calif.) was prepared in rabbits immunized with carrier keyhole limpet hemocyanin (KLH, Calbiochem) conjugated to FITC at a molar ratio of 45:1. The IgG fraction of the antiserum to the KLH-FITC complex was made specific for the FITC hapten by absorption with the KLH carrier immobilized on agarose by the cyanogen bromide method (15). The anti-FITC antibody was further purified by absorption and elution from FITC-human IgG conjugated to agarose. The anti-FITC was eluted by incubation with 3 M NH_4SCN for 20 min at room temperature, and then dialyzed against phosphate-buffered saline (PBS), pH 7.5, at 4 °C overnight. The eluted anti-FITC antibody demonstrated a precipitin line when tested by immunodiffusion against FITC-conjugated KLH or human IgG-FITC, but showed no detectable activity against KLH or IgG alone.

IgG, IgG fragments, and different-sized aggregates of IgG. IgG fractions of rabbit anti-FITC, anti-C3, anti-IgM, anti-IgA, and anti-IgD were prepared by column chromatography of the antisera on DEAE-cellulose (DE-52, Whatman Chemicals, Div. W & R Balston, Maidstone, Kent, England) in 0.0175 M phosphate buffer, pH 7.0. The Fab and Fc fragments from the anti-FITC, anti-IgM, and anti-IgD were prepared by papain digestion and carboxymethylcellulose (CM-52, Whatman Chemicals) column chromatography by a modification of the method of Porter (16). The Fab fragments were further purified by absorption with protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) to remove traces of whole and partially cleaved IgG. Fab and IgG contamination was removed from the Fc fragments by recrystallization three times. The $F(ab')_2$ and pFc fragments were prepared from anti-IgA and anti-C3 by pepsin digestion according to a modification of the method of Nisonoff et al. (17). The $F(ab')_2$ and pFc fragments were separated by Sephadex G-150 (Pharmacia Fine Chemicals) column chromatography. Trace amounts of uncleaved IgG were removed from the $F(ab')_2$ fragments by passage through protein A-Sepharose CL-4B (Pharmacia Fine Chemicals).

Each of the various IgG fragments was tested for antibody activity versus the homologous antigen. When examined by passive hemagglutination with IgA or C3 coated erythrocytes, the bivalent $F(ab')_2$ fragments had exactly the same antibody activity on a molar basis as did the parent IgG molecules. The monovalent Fab fragments derived from anti-FITC, anti-IgM, and anti-IgD were fully active in inhibiting the agglutination of antigen coated erythrocytes by native bivalent antibody. Neither the Fc fragments nor the pFc fragments had any detectable antibody activity.

The rabbit IgG antibody preparations (anti-FITC, -C3, -IgM, -IgA, and -IgD) were homogenous on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis performed by the method of Weber and Osborn (18) and gave a single precipitin line of identity in Ouchterlony immunodiffusion with goat anti-rabbit IgG and goat anti-rabbit serum (N.L. Cappel Laboratories Inc., Cochranville, Pa). The $F(ab')_2$ fragments prepared from the anti-C3 and the anti-IgA showed no traces of whole IgG on SDS-polyacrylamide gel electrophoresis. The Fab and Fc fragments purified from the anti-FITC, anti-IgD, and anti-IgM gave lines of nonidentity when tested in Ouchterlony immunodiffusion against goat anti-rabbit IgG. In addition, SDS-polyacrylamide gel electrophoresis of the unreduced fragments showed no contamination of the Fab with Fc nor contamination of the Fc with Fab.

Bence Jones λ-light chains were prepared from the urine of a patient with multiple myeloma by precipitation with 40% ammonium sulfate followed by two cycles of Sephadex G-100 (Pharmacia Fine Chemicals) column chromatography.

Purified human IgG was prepared from normal serum by chromatography on DE-52 cellulose (0.01 M sodium phosphate, pH 7.0) followed by Sephadex G-200. Part of this IgG preparation, 200 mg in 4 ml of PBS, was heat aggregated at 63 °C for 10 min and then layered onto a 2.5× 100 cm column of Sepharose CL-6B (Pharmacia Fine Chemicals) equilibrated with 1.0 M NaCl in 0.05 M Tris-citrate, pH 8.0. The molecular weights of various aggregate containing fractions were determined by comparing the elution volume to void volume ratio to that previously determined for known molecular weight markers. The majority of IgG was contained in either very large aggregates (>2 × 10^6 mol wt) or monomeric IgG (150,000 mol wt), and only 7.2 mg of IgG was isolated in fractions containing 300,000–450,000 mol wt aggregates (IgG dimers and trimers). Aggregate containing fractions were concentrated by dehydration in dialysis tubing covered with granular sucrose, followed by dialysis against PBS.

Radioiodination of IgG aggregates. A pool of human IgG dimers and trimers, prepared freshly as described above, was radioiodinated with insolubilized lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) and hydrogen peroxide as described by David and Reisfeld (19). The ^{125}I-labeled heat-aggregated IgG contained 71,000 cpm/μg protein.

Sources of von Willebrand factor. Three sources were

[1] *Abbreviations used in this paper*: FITC, fluorescein isothiocyanate; FWP, formalin-fixed washed platelets; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SDS, sodium dodecyl sulfate.

used for von Willebrand factor: Hemofil (Hyland Laboratories, Costa Mesa, Calif.), Profilate (Abbott Diagnostic, Diagnostic Products, North Chicago, Ill.) and, for selected experiments, von Willebrand factor purified from Hemofil (kindly provided by Dr. Eric Jaffe of Cornell University Medical College, New York). In the system described below for testing the agglutination of FWP by von Willebrand factor and ristocetin, the minimum final concentration of the various preparations which supported ristocetin-induced agglutination was: Hemofil, 3.86 μg/ml, Profilate, 0.370 μg/ml, and purified von Willebrand factor, 0.038 μg/ml. The purified von Willebrand factor, which was ≅95% pure on SDS-gel electrophoresis (20) was the same preparation used to follow Factor VIII antigen in the sucrose density gradient ultracentrifugation studies described below.

Agglutination studies with standard aggregation module. 0.45 ml vol of PRP were stirred at 37 °C in siliconized cuvettes in a Payton aggregation module (Payton Associates, Buffalo, N. Y.), and aggregating agents diluted in Tris-buffered saline, pH 7.35, were added. The responses were recorded on a Riken Denshi linear recorder (Payton Associates). For the studies of the inhibition of ristocetin agglutination, 40 μl of the test material or Tris-saline (control) was added to PRP at a stated time interval before ristocetin was added. In addition to the IgG and IgG fragments, purified human ceruloplasmin and bovine serum albumin (Sigma Chemical Co.) were used as controls. Ristocetin, 10 mg/ml (Abbott Diagnostics, Diagnostic Instruments, South Pasadena, Calif.), was added to the reaction mixture to give a final concentration of 1.09 mg/ml. ADP and collagen (Sigma Chemical Co.) were added to duplicate controls at the lowest concentrations which caused reproducible aggregation.

Agglutination studies with a microtiter plate. 0.225 ml vol of PRP or FWP resuspended in PPP were placed in the rounded well (1.6 ml capacity) of a siliconized-Lucite (E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.) microtiter plate. 20 μl of Tris-saline (control well) or of the preparation to be tested was added followed by 30 μl of ristocetin, 10 mg/ml (final concentration, 1.09 mg/ml). The plate was agitated by hand at room temperature for 3–5 min and the agglutination was recorded by visual and microscopic inspection with an inverted microscope.

Agglutination studies in a plasma-free system. 37.5 μl of a serially diluted sample of von Willebrand factor (Hemofil, Profilate, or purified Factor VIII), 37.5 μl of the protein to be tested (bovine serum albumin, rabbit IgG, monomeric or aggregated human IgG, Fc fragment, Fab fragment, or pFc fragment, each at a final protein concentration of 0.7 mg/ml), 25 μl of ristocetin (final concentration, 0.3–0.4 mg/ml) and 100 μl of FWP in PBS (final concentration, 100,000/μl) were added to the well of a siliconized microtiter plate according to a modification of the method of Weksler et al. (21). The plate was agitated on a rotary shaker for 20 min at room temperature, and the agglutination recorded as above.

Serial dilutions of Fc fragments were also tested for inhibition of ristocetin agglutination in a similar system that incorporated fixed amounts of von Willebrand factor.

Sucrose density gradient ultracentrifugation of von Willebrand factor, ristocetin, and aggregated IgG. 5 μl of ^{125}I-labeled heat-aggregated IgG (325 μg/ml), consisting of dimers and trimers as determined by the method described above, were added to 75 μl of ristocetin, 10 mg/ml, and 25 μl of purified von Willebrand factor, 710 μg/ml in PBS. After 10 min at room temperature, the sample was applied to a continuous 4.6-ml density gradient prepared with 10–40% sucrose in 0.01 M PBS, pH 7.4, and centrifuged at 32,000 rpm (122,000 *g*) for 18 h at 4°C in a Beckman SW-50 Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Control gradients run simultaneously included von Willebrand factor alone, von Willebrand factor and ristocetin, ^{125}I-labeled heat-aggregated IgG alone, ^{125}I-labeled heat-aggregated IgG and von Willebrand factor, and ^{125}I-labeled heat-aggregated IgG and ristocetin. After ultracentrifugation, the gradients were fractionated by piercing the bottom of the tubes and collecting 0.17–0.18-ml samples. The Factor VIII material in the gradient fractions was followed by quantitating the Factor VIII antigen levels with a modification of the Laurell quantitative immunoelectrophoretic technique as described by Zimmerman et al. (22). ^{125}I-Aggregated IgG was quantitated by measuring the radioactivity in 25-μl samples of each fraction.

Function of von Willebrand factor after incubation with aggregated IgG and ristocetin. Cohn fraction II (Miles Laboratories Inc., Elkhart, Ind.), was heat aggregated at 63°C for 15 min and conjugated to Bio-Gel A-5m agarose (Bio-Rad Laboratories, Richmond, Calif.) by the cyanogen bromide method at a ratio of 4 mg protein to 1 ml of gel. The gel was tested for the availability of the Fc region of IgG by incubating 200 μl of gel with 1 drop of FITC-labeled staphylococcal protein A (kindly provided by Dr. E. Rabellino, Cornell University Medical College, New York). The protein A binds specifically to the Fc region of IgG (23). After 30 min at room temperature, the gel was washed three times and examined for fluorescence with a Leitz Ortholux II microscope equipped with a Ploem illuminator (E. Leitz Inc., Rockleigh, N. J.).

1 ml of the aggregated IgG-gel was placed in a 1.8 × 8-cm column and equilibrated with PBS that contained 0.5 mg/ml of ristocetin and 1% human serum albumin (Miles Laboratories Inc.). Von Willebrand factor (Profilate, 140 μg in 200 μl) was placed on the column and eluted after 10 min with the ristocetin-albumin buffer into 0.5-ml fractions. 100-μl samples of each eluted fraction were tested for von Willebrand-factor activity by a modification of the microtiter plate assay (21) and compared to similar fractions eluted from a control column of Bio-Gel A-5m agarose not conjugated to IgG.

Fluorescence assay for platelet Fc receptors. Platelets were washed according to the method of Pfueller et al. (12), and labeled with soluble fluorescent immune complexes (KLH-anti-KLH) to detect the Fc receptor. The assay was a modification of a procedure described by Abbas and Unanue (24) for detection of lymphocyte Fc receptors. Both the antigen and the antibody were conjugated to FITC (1–2 μg FITC/mg protein). A pellet of 1×10^8 washed platelets was resuspended in a mixture of 25 μl of KLH (100 μg/ml) and 25 μl of anti-KLH (3 mg/ml), and incubated at room temperature for 30 min. The platelets were washed once by centrifugation through a 3-ml gradient of 2–4% bovine serum albumin in PBS at 600 *g* for 15 min. The pellet was resuspended and examined for fluorescence.

The fluorescent immune complexes stained ≅80–90% of normal platelets. With the majority of platelets, the staining pattern was "patchy" with one to three very fine fluorescent dots per platelet, and only an occasional platelet was stained in a crescent pattern that outlined a portion of the platelet periphery. With the method outlined above, excessive platelet clumping did not occur although there was occasional microscopic clumping which made exact quantitation difficult. The specificity of the staining reaction for Fc receptors was demonstrated by the inhibition of the staining reaction produced by prior incubation of the platelets with purified Fc fragments, 3 mg/ml. In addition, there was no platelet fluorescence-staining produced with either the FITC-conjugated antigen or antibody alone.

Inhibition of platelet Fc receptor staining by von Willebrand factor and ristocetin. 1×10^8 washed platelets were suspended in 70 μl of PBS which contained von Willebrand factor (Hemofil, 2.5 μg to 1 mg/ml) and 0.6 mg/ml ristocetin.

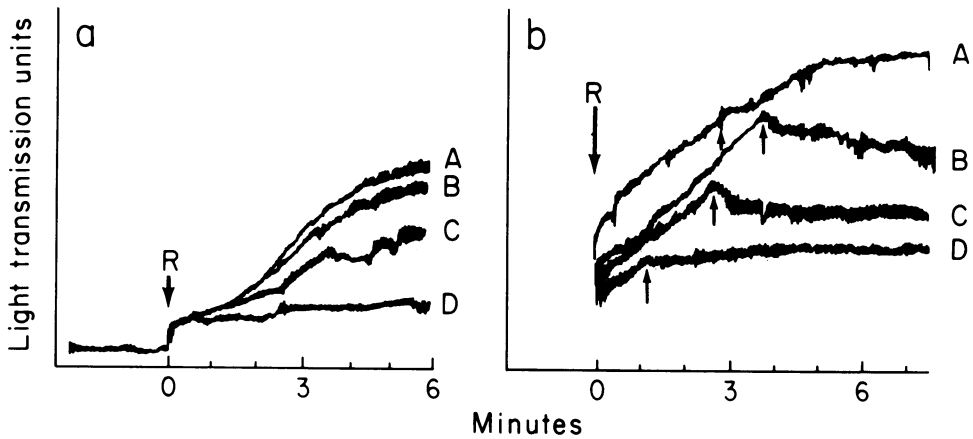

FIGURE 1 Effect of whole rabbit antiserum on ristocetin-induced agglutination in PRP. (*a*) An increasing of inhibition of platelet agglutination with increasing amounts of whole rabbit antiserum (anti-$\alpha_2$macroglobulin). Antiserum added 5 min before ristocetin (R). A: Tris-saline; B: 20 μl antiserum; C: 30 μl antiserum; D: 50 μl antiserum. (*b*) Interruption of platelet agglutination by whole rabbit antiserum (anti-human IgM). A: Tris-saline added at 3 min (arrow); B, C, and D: antiserum added at 4, 3, and 1 min, respectively (arrows).

Platelet agglutination was recorded after intermittent agitation for 10 min. Control preparations without ristocetin or without von Willebrand factor were tested simultaneously. In this system, the lowest concentration of von Willebrand factor which gave gross or microscopically detectable clumping in the presence of ristocetin was 5 μg/ml. The platelet preparations were then centrifuged at 1,000 *g* for 20 min, the supernate removed, and the platelets in the pellet resuspended and reacted with the KLH-anti-KLH reagent as described above.

RESULTS

Inhibition of ristocetin-induced agglutination by whole rabbit antisera. Ristocetin-induced agglutination of platelets in PRP was blocked by the prior addition of a variety of different whole rabbit antisera (e.g., anti-IgG, -albumin, -IgM, -fibrinogen, -ceruloplasmin, or -α_2-macroglobulin) but not by bovine serum albumin, or purified human ceruloplasmin. Blocking was dose dependent (Fig. 1*a*), and occurred rapidly, even when the ristocetin was added immediately after the antiserum with no preincubation. Once the ristocetin-induced agglutination had started, it could be interrupted and partially reversed by the addition of an aliquot of antiserum (Fig. 1*b*). Platelet aggregation with other aggregating agents (e.g., ADP or collagen) was not inhibited by any of the whole rabbit antisera examined. To test whether the blocking factor(s) might be either aggregated IgG or immune complexes contained in these antisera, further experiments were performed with purified, normal human monomeric and aggregated IgG as well as various fragments of rabbit IgG antibody.

Inhibition of ristocetin-induced agglutination by pure rabbit IgG, the Fc fragments, and aggregated human IgG. IgG (final concentration, 0.1–1 mg/ml) purified from rabbit antisera specific for human IgA, human IgD, or FITC, blocked ristocetin-induced agglutination of platelets in PRP. The F(ab')$_2$ fragment prepared from the antiserum to IgA, and the Fab and Fc fragments prepared from the antiserum to FITC, were tested at final concentrations of 0.07 mg/ml (Fig. 2). The Fc fragment blocked ristocetin-induced agglutination while the Fab and F(ab')$_2$ fragments did not (Fig. 2).

Isolated rabbit IgG and Fc fragments also blocked the ristocetin-induced agglutination of fixed, washed platelets suspended in PPP. The Fab fragments from the same IgG preparations and purified λ-light chains did not block this agglutination response. Although the data are not shown, inhibition was also obtained with Fc fragments from both anti-IgM and anti-IgD, while

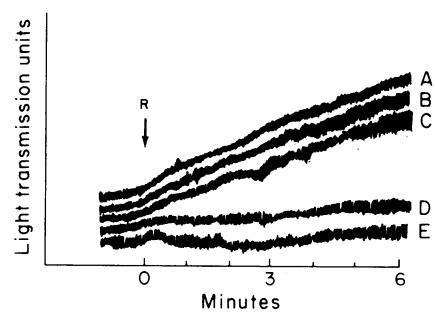

FIGURE 2 Effect of pure IgG and IgG fragments on ristocetin-induced agglutination in PRP. Tris-saline or protein (final concentration, 0.07 mg/ml) was added to the PRP 1 min before the ristocetin (R). A: Tris-saline; B: Fab fragment of anti-FITC; C: F(ab')$_2$ fragments of anti-IgA; D: intact IgG anti-FITC; E: Fc fragment of anti-FITC.

TABLE I

*Inhibition of Ristocetin-Induced FWP Agglutination in Plasma by Fc and Aggregated IgG**

Agglutination after the addition of:	Agglutination
Tris-saline	++++
Whole IgG‡ (0.3 mg/ml, final concentration)	0
Fab fragments (0.3 mg/ml)	++++
Fc fragments (0.3 mg/ml)	0
λ-Chains (1 mg/ml)	++++
Human IgG, freed of aggregates (0.6 mg/ml)	++++
Heat-aggregated human IgG (0.6 mg /ml)	0

* This experiment was performed in a microliter plate with plasma as the source of von Willebrand factor (final concentration of ristocetin, 1.09 mg/ml). The grading system is: zero, no microscopic clumps; trace, microscopic clumps only; +, many small clumps; ++, many medium-sized clumps; +++, two to four large clumps; ++++, one large clump.

‡ Unless otherwise noted the IgG and IgG fragments used in these experiments were derived from rabbit anti-FITC serum.

the Fab fragments from these two antibodies, as well as the $F(ab')_2$ fragments from anti-C3, were noninhibitory. Purified human IgG, freed of aggregates by Sephadex G-200 column chromatography, did not block the ristocetin-induced agglutination of platelets. However, after heat aggregation at 63°C for 10 min, this same preparation of human IgG became a potent inhibitor of ristocetin-induced agglutination (Table I).

Reciprocal effects of von Willebrand factor and Fc concentrations on ristocetin agglutination. In a plasma-free system, the highest dilution of von Willebrand factor which supported ristocetin-induced platelet agglutination was 1:2048. This represented a final concentration of 3.86 μg/ml of Hemofil, 0.370 μg/ml of Profilate, or 0.038 μg/ml of purified von Willebrand factor. At this dilution, platelet agglutination was not effected by PBS, bovine serum albumin, Fab or pFc fragments of rabbit IgG, or monomeric human IgG. However, in the presence of rabbit IgG (not specifically freed of aggregates), Fc fragments, or aggregates of human IgG ranging from small dimers (300,000 mol wt) up to large aggregates ($>2 \times 10^6$ mol wt) an 8- to 16-fold increase in the concentration of von Willebrand factor was required to support ristocetin-induced platelet agglutination (Table II). When a constant amount of von Willebrand factor was used in this system, Fc fragments produced a dose-dependent inhibition of ristocetin-induced platelet agglutination (Table III).

Absence of IgG-ristocetin-von Willebrand factor complex formation in the fluid phase. To determine whether aggregated IgG inhibited the agglutination of platelets by forming a complex with von Willebrand factor and ristocetin in the fluid phase, sucrose density gradient analysis was performed with ^{125}I-labeled heat-aggregated IgG before and after incubation with ristocetin and von Willebrand factor. The von Willebrand factor was followed by the location of immunoprecipitable Factor VIII antigen. Studies by others have shown that von Willebrand factor and Factor VIII antigen move together in sucrose density gradients in the presence or absence of ristocetin (25). Dimers and trimers of IgG which were previously shown to inhibit von Willebrand factor and ristocetin-induced agglutination of platelets, were selected for ^{125}I-labeling because aggregates of this size were relatively small compared to von Willebrand factor/Factor VIII antigen and would sediment a much smaller distance than Factor VIII antigen in the sucrose density gradient. Therefore, if any complex formed between the aggregated IgG and von Willebrand factor in the presence or absence of ristocetin, it would be easily detectable by a shift of the radioactivity down the gradient toward the Factor VIII antigen.

The results of these experiments (Fig. 3) demonstrated that Factor VIII antigen was distributed into

TABLE II

*Inhibition of von Willebrand Factor and Ristocetin-Induced Platelet Agglutination by Aggregated IgG and Fc Fragments in a Plasma-Free System**

Agglutination after the addition of:	Dilution of von Willebrand factor‡				
	1:128	1:256	1:512	1:1,024	1:2,048
PBS, bovine serum albumin, monomeric human IgG, Fab, or pFc (0.7 mg/ml, final concentration)	+++§	++	++	+	Trace
Rabbit IgG or heat-aggregated human IgG‖ (0.7 mg/ml)	++	Trace	0	0	0
Rabbit Fc (0.7 mg/ml)	+++	0	0	0	0

* This experiment was performed in a microtiter plate with FWP in PBS (final concentration of ristocetin 0.4 mg/ml).

‡ The final concentration of von Willebrand factor in undiluted well was: Hemofil 7.9 mg/ml, Profilate 760 μg/ml, or purified Factor VIII 78 μg/ml.

§ The grading system is the same as in Table I.

‖ Eight preparations of increasing molecular weights ($3 \times 10^5 - >2 \times 10^6$) as determined by Sepharose CL-6B column chromatography were tested. All preparations were inhibitory.

TABLE III

Fc Fragment Inhibition of Ristocetin-Induced Agglutination of FWP in a Plasma-Free System

Fc*, mg/ml	0.7	0.5	0.4	0.2	0.15
Agglutination in the presence of von Willebrand factor (Profilate, 1.45 μg/ml)‡	0	+	++	+++	++++

* Final protein concentration in the separate test systems was kept constant at 0.7 mg/ml with bovine serum albumin.
‡ The grading system is the same as in Table I.

fraction 3 through 12 with the peak at fraction 8. This pattern was not changed by the presence of ristocetin (Fig. 3*a*). The labeled aggregates of IgG were localized to fractions 9 through 27 with the peak at fraction 16. This pattern was unchanged by prior mixing of the aggregates IgG with von Willebrand factor, ristocetin, or a mixture of von Willebrand factor and ristocetin (Fig. 3*b* and *c*).

Retention of von Willebrand factor activity after incubation of von Willebrand factor with aggregated IgG and ristocetin. After elution of von Willebrand factor from aggregated IgG-agarose in the presence of ristocetin, von Willebrand factor activity was found to be identical to that eluted from the control agarose column. In both column eluates, von Willebrand factor activity was found in the first four fractions. When the four fractions from each column were pooled, the highest dilution of the pooled fractions which had von Willebrand factor activity was 1:8.

The aggregated IgG-agarose used in these experiments had available Fc regions as demonstrated by the fluorescence staining obtained with FITC-labeled protein A. FITC-labeled protein A did not stain the unconjugated agarose.

Inhibition of platelet Fc receptor staining by von Willebrand factor and ristocetin. Platelet Fc receptor staining by the KLH-anti-KLH complex was almost completely abolished by prior incubation of the platelets with barely agglutinating concentrations of von Willebrand factor (Hemofil, final concentration 5–10 μg/ml) and ristocetin (final concentration, 0.6 mg/ml) (Table IV). Incubation of platelets with varying concentrations of von Willebrand factor alone or with ristocetin alone did not affect subsequent platelet staining. If the platelets were washed in PBS one time after their interaction with an inhibitory concentration of von Willebrand factor and ristocetin, their ability to bind fluorescent immune complexes was restored.

DISCUSSION

These studies suggest that there is a close relationship between the platelet receptor for the Fc region of IgG and the surface glycoprotein I complex that interacts with von Willebrand factor in the presence of ristocetin to produce platelet agglutination. The von Willebrand factor-dependent ristocetin-induced platelet agglutination reaction was blocked by aggregated IgG and by purified Fc fragments of IgG, but not by monomeric IgG, $F(ab')_2$, Fab, or pFc fragments. This inhibitory activity of IgG was unrelated to its antigenic specificity, because with each rabbit antibody examined, inhibitory activity was restricted to the Fc portion of the molecule, whereas the antibody activity was fully preserved in the noninhibitory Fab or $F(ab')_2$ portion. Platelet Fc receptors were detected by fluorescence microscopy after incubation of platelets with fluorescent soluble complexes of IgG antibody and antigen. This fluorescence reaction, which was blocked by prior treatment of platelets with purified Fc fragments, was also blocked by treating platelets with von Willebrand factor in the presence of ristocetin.

To test whether aggregated IgG or purified Fc fragments interfered with ristocetin or von Willebrand factor in the fluid phase rather than at the platelet surface, sucrose density gradient ultracentrifugation analysis was performed on ^{125}I-labeled heat-aggregated IgG both before and after incubation with von Willebrand factor and ristocetin. With similar techniques, Floyd et al. (26) described a significant aggregation of von Willebrand factor by ristocetin. Our results showed no detectable complex formation between von Willebrand factor and ristocetin and no interaction between aggregated IgG and either von Willebrand factor or ristocetin, alone or in combination. The function of von Willebrand factor was also unaffected by prior incubation with a mixture of ristocetin and aggregated IgG.

Both the Fc fragment interference in the ristocetin-agglutination reaction, and the von Willebrand factor and ristocetin blockade of the platelet Fc receptor appeared to occur at the level of the platelet membrane. The finding that von Willebrand factor alone was not capable of blocking the Fc receptor is consistent with the hypothesis that ristocetin is required for von Willebrand factor binding to platelets (27, 28). The platelet Fc receptor and the platelet surface protein(s) which mediate the von Willebrand factor and ristocetin agglutination reaction are apparently not identical. The surface protein(s) which mediates ristocetin-induced von Willebrand factor-dependent platelet agglutination can be removed by digestion of the platelet with chymotrypsin whereas the platelet Fc receptor is insensitive and even demonstrates enhanced binding of aggregated IgG after mild proteolytic digestion (6, 29).

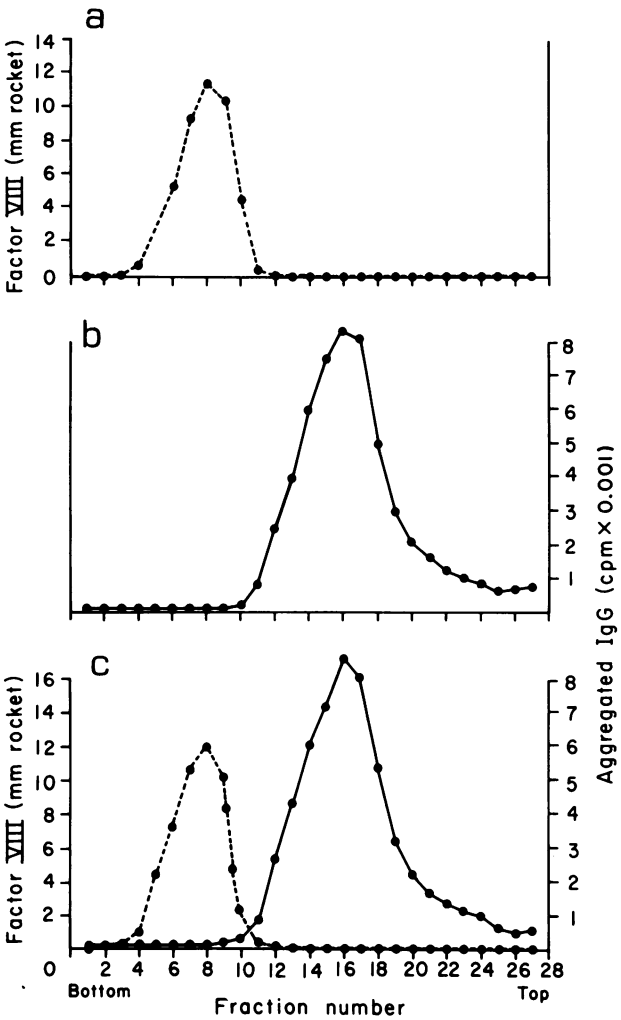

FIGURE 3 Sucrose density gradient ultracentrifugation analysis of Factor VIII antigen measured by the height of the immunoprecipitin rocket (● - - - ●), and ^{125}I-labeled heat-aggregated IgG (● —— ●). The amounts placed on the gradients were: 5 μl ^{125}I-labeled heat-aggregated IgG, 325 μg/ml (predominantly dimers and trimers); 75 μl ristocetin, 10 mg/ml; 25 μl purified Factor VIII antigen, 710 μg/ml. Factor VIII refers to Factor VIII antigen. Panel *a* shows the sedimentation behavior of Factor VIII antigen after incubation with ristocetin. This pattern is identical to that obtained with Factor VIII antigen alone. Panel *b* shows the distribution of ^{125}I-labeled heat-aggregated IgG after incubation with ristocetin. The pattern is identical to that obtained with aggregated IgG alone and with aggregated IgG incubated with Factor VIII antigen. Panel *c* shows the distribution of factor VIII antigen and aggregated IgG after incubation of a mixture which contained factor VIII antigen, aggregated IgG, and ristocetin.

Occupation of the Fc receptor by aggregated IgG may mask the surface structure which mediates the ristocetin-induced von Willebrand factor-dependent platelet reaction and thus interfere with subsequent platelet agglutination. Similarly, ristocetin-induced occupation of the surface glycoprotein I complex on the platelet membrane by the von Willebrand factor may mask the Fc receptor and interfere with the binding of immune complexes. The fact that the blocking reaction occurs with formalin-fixed platelets rules out membrane fluidity changes as a cause for these surface relationships. The data suggest that the platelet Fc receptor may be either part of the surface glycoprotein I complex or sterically related to it, and that occupation of one of these surface components hinders the availability of the other.

TABLE IV
Inhibition of Platelet Fc Receptors Staining by von Willebrand Factor and Ristocetin

Treatment of platelets before incubation with immune complex	Fluorescent Fc staining*
PBS	3+
Fc fragments (3 mg/ml)	0
Von Willebrand factor (Hemofil, 5–10 μg/ml)	3+
Ristocetin (0.6 mg/ml)	3+
Von Willebrand factor and ristocetin	0–1+

* The grading refers to the numbers of platelets stained. 3+ = 80–90% of platelets stained with one to three fine dots per platelets; 1+ = 1–5% of platelets stained.

The observation that either IgG, Fc fragments, or aggregates of IgG as small as dimers profoundly interfere with the agglutination of platelets by ristocetin and the von Willebrand factor is of great importance to those studies that use immunologic probes to explore this reaction (6, 30–32). Studies that demonstrate interference of this reaction obtained with whole antisera or isolated IgG might require confirmation by experiments with the Fab or F(ab')$_2$ fragments alone, as our results suggest that most rabbit antisera contain enough aggregated IgG or immune complexes to nonspecifically interfere with the agglutination reaction. In addition, the finding that occupation of the platelet Fc receptor blocks the reaction of ristocetin and von Willebrand factor with the platelet surface suggests the possibility that there may be some acquired abnormalities of ristocetin-induced platelet agglutination in clinical conditions associated with circulating immune complexes. In preliminary studies, we have observed absent platelet agglutination in response to ristocetin in the platelet-rich plasma of 4 of 12 patients with systemic lupus erythematosus.[2] The refractoriness to ristocetin in such patients may be a result of occupation of the platelet Fc receptor by circulating immune complexes with subsequent masking of the von Willebrand factor receptor site.

[2] Moore, A. Unpublished observations.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Michael Lockshin for his referral of the patients with systemic lupus erythematosus, and to Ms. Reneé Zwiebel-Posner and Ms. Leslie Goodwin for their excellent technical assistance.

This work was supported by National Institutes of Health grant CA-16190, Specialized Center for Research in Thrombosis grant HL-18828 from the National Institutes of Health, as well as by the Arnold Krakower Foundation.

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