JCI The Journal of Clinical Investigation

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J Clin Invest. 1976;57(1):112-124. https://doi.org/10.1172/JCI108251.

Research Article

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Platelet Membrane Glycoproteins Implicated in Ristocetin-Induced Aggregation

STUDIES OF THE PROTEINS ON PLATELETS FROM PATIENTS WITH BERNARD-SOULIER SYNDROME AND VON WILLEBRAND'S DISEASE

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ABSTRACT The antibiotic ristocetin only aggregates platelets in the presence of plasma von Willebrand factor. Platelets from patients with Bernard-Soulier syndrome do not aggregate upon addition of ristocetin although, in contrast to von Willebrand's disease, plasma levels of factor VIII complex (factor VIII clotting activity, von Willebrand factor activity, and von Willebrand antigen) are normal.

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Staining of gels for protein and carbohydrate indicated that there was an extensive change in the surface of Bernard-Soulier platelets, whereas those from patients with von Willebrand's disease appeared the same as normal. Platelets from patients were labeled by the lactoperoxidase iodination technique. Not only was the relative intensity of staining of platelet-specific proteins and glycoproteins changed in Bernard-Soulier platelets, but the iodination of the glycoproteins on the membrane surface relative to other membrane constituents was lower. In contrast, platelets from patients with von Willebrand's disease showed a normal exposure of membrane components. These data suggest therefore that membrane glycoproteins may play a functional role in ristocetin-induced aggregation.

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Received for publication 8 April 1975 and in revised form 25 August 1975.

INTRODUCTION

Ristocetin-induced aggregation of platelets and platelet adhesion to the subendothelial surface of blood vessels are abnormal in two bleeding disorders, von Willebrand's disease (1–4) and Bernard-Soulier syndrome (5–7). In von Willebrand's disease the abnormality is due to the lack of a plasma factor, von Willebrand factor, which is part of the factor VIII complex. Patients with Bernard-Soulier syndrome have normal levels of von Willebrand factor because their plasma corrects abnormal ristocetin-induced aggregation in von Willebrand's disease (5, 6). Thus, the primary defect in this disorder would appear to be associated with the platelet.

The mechanism of ristocetin-induced aggregation is unknown but requires both a receptor on the platelet and von Willebrand factor (8). It is not known if von

Willebrand factor and ristocetin form a complex or if von Willebrand factor is modified by ristocetin so that it acquires aggregating properties. Although ristocetin-induced aggregation is a nonphysiological phenomenon, it nevertheless may be a useful tool for investigating the initial events of primary hemostasis.

It has recently been shown that one ristocetin preparation (lot 3) contains a proteolytic contaminant (9): the action of this proteolytic enzyme on normal platelets rendered them less responsive to ristocetin-induced aggregation presumably by action on the receptor on the platelet.[1] Since the platelets from patients with Bernard-Soulier syndrome do not respond to ristocetin (5, 6) it appeared of considerable interest to determine whether the receptor affected by proteolysis was the same as that which shows abnormalities in this pathological condition. For this purpose an investigation of the exposed proteins of the plasma membrane of these platelets was undertaken using the lactoperoxidase iodination technique (10-12), together with studies involving conventional staining techniques. The results obtained were compared with those obtained with normal platelets modified by proteolysis. Furthermore, platelets from two patients with von Willebrand's disease were included in the present study.

METHODS

Isolation of platelets. Six parts of blood from normal donors and patients were collected in plastic tubes containing one part of acid citrate dextrose (ACD)[2] (13). Blood from normal donors and from patients with von Willebrand's disease was centrifuged at 160 *g* for 10 min at room temperature to obtain platelet-rich plasma (PRP), and the platelets were washed by either the method of Massini and Lüscher (14) or according to Jenkins et al. (8). The method of Massini and Lüscher involved separation of platelets from plasma by differential centrifugation followed by three washing steps: platelets were washed twice using 0.12 M sodium chloride, 0.0129 M sodium citrate, 0.03 M glucose, and once with 0.154 M sodium chloride, 0.01 M Tris, 1 mM EDTA, pH 7.4, and resuspended in this buffer. Platelets washed by this method aggregated to 2 mg/ml ristocetin and thus could not be used in experiments involving the effects of different ristocetin preparations on platelets. Platelets for such a study were washed according to Jenkins et al. (8): this involved four washing steps using 0.154 M sodium chloride, 0.0154 M Tris, 5.5 mM glucose, 6.6 mM EDTA, pH 7.35 as washing fluid, and the platelets were then resuspended in 0.154 M sodium chloride, 0.0154 M Tris, 5.5 mM glucose, pH 7.35. Platelets washed in this way demonstrated no aggregation by ristocetin unless a source of von Willebrand factor was added.

Isolation of platelets from the patient with Bernard-Soulier syndrome presented technical difficulties due to similarity in size of the platelets to white cells. Conventional differential centrifugation of blood from this patient resulted in a platelet suspension containing a high percentage of white cells. Two methods for platelet isolation were employed and compared for platelet yield and purity. The first method required that the blood be left to settle at room temperature for 1 h. The PRP was decanted and centrifuged, and the platelets were washed three times according to Massini and Lüscher (14). Each washing step included a differential centrifugation at 80 *g* for 2 min. The final suspension (30% yield) contained 10^9 platelets/ml with 15% white cell contamination (control: 10^9 platelets, 1% white cell contamination). In the second method, PRP was obtained as described above, and the platelets were separated from other blood constituents by the technique of Mohammad et al. (15), involving their aggregation with ADP before washing. PRP was treated with ADP 10 μM final concentration, and the aggregates were sedimented by centrifugation at 100 *g* for 30 s. The aggregates were rinsed twice with 0.154 M sodium chloride without disturbing the pellet and were disaggregated with 0.154 M sodium chloride, 2 mM calcium chloride containing 1 mg/ml potato apyrase. The mixture was incubated at 37°C for 10 min, and the platelets were sedimented by centrifugation at 2,700 *g* for 20 min. The platelets were suspended in 0.3 ml 0.154 M sodium chloride and diluted by the addition of 0.154 M sodium chloride, 0.01 M Tris, 1 mM EDTA, pH 7.4. The final suspension (30% yield) contained 10^9 platelets/ml with 1% white cell contamination (control: 10^9 platelets, 0.5% white cell contamination).

Treatment of washed normal platelets with proteases. 1-ml platelets (10^9 platelets) from normal donors, washed by the method of Jenkins et al. (8), were incubated at 37°C for 3 min before the addition of 10 μl 0.154 M sodium chloride containing: (*a*) 0.154 M sodium chloride alone; (*b*) 2 mg ristocetin lot 3003-109-30 (protease free); (*c*) 2 mg ristocetin lot 3 (containing protease contaminant [s]); (*d*) 2 mg ristocetin lot 3 previously incubated with 0.05 M diisopropyl fluorophosphate (DFP) for 16 h at 4°C; or (*e*) 0.05 M DFP previously incubated for 16 h at 4°C. The samples were incubated for an additional 5 min after which they were centrifuged at 2,700 *g* for 15 min. The platelet pellets were resuspended in 0.4 ml water and solubilized by the addition of 0.2 ml 10% sodium dodecyl sulfate (SDS) as previously described (11). 1-ml platelets (10^9 platelets) from normal donors, washed by the method of Jenkins et al. (8), were also incubated at 37°C for 15 min with 10 μl 0.134 M sodium chloride, 0.01 M sodium phosphate, pH 7.35, containing: (*a*) buffered solution alone; (*b*) 10 μg chymotrypsin; (*c*) 50 μg chymotrypsin; or (*d*) 100 μg chymotrypsin. Chymotrypsin solutions were prepared immediately before use. The platelets were sedimented by centrifugation and washed three times with 0.154 M sodium chloride, 0.0154 M Tris, 6.6 mM EDTA, pH 7.4. Aliquots of these washed chymotrypsin-treated platelet suspensions were then tested for aggregation after addition of normal plasma and ristocetin: the remaining platelets were sedimented by centrifugation and solubilized as described above.

Aggregation studies. Platelet aggregation was studied at 37°C by the turbidimetric method of Born (16) using a Labintec aggregometer (Montpellier, France). A 0.2-ml sample of a platelet suspension (2×10^8 platelets/ml), was treated with 0.04 ml 0.154 M sodium chloride or a standard pool plasma (20 donors, stored at −70°C [17]), and incubated for 1 min in the aggregometer before the addition of 0.01 ml ristocetin lot 3003-109-30. Ristocetin was at a final concentration of 2 mg/ml.

[1] Jenkins, C. S. P., D. Meyer, N. Ardaillou, C. Lombard, and M-J. Larrieu. To be published.

[2] *Abbreviations used in this paper:* ACD, acid citrate dextrose; DFP, diisopropyl fluorophosphate; PRP, platelet-rich plasma; SDS, sodium dodecyl sulfate.

Protein determination. Protein concentration was determined by the method of Lowry et al. (18).

Patients. Three patients, two fulfilling the diagnostic criteria of von Willebrand's disease (17) and the other of Bernard-Soulier syndrome (19), were studied. The patients with von Willebrand's disease had long Ivy bleeding times (> 15 min, normal range 4–8 min), low levels of factor VIII complex[3] (patient 1: factor VIII clotting activity 37%, von Willebrand antigen 38%, von Willebrand factor activity < 5%; patient 2: factor VIII activity < 5%, von Willebrand antigen < 5%, von Willebrand factor activity < 5%, normal range 50–200% [17]), absence of ristocetin-induced aggregation, and normal aggregation to bovine von Willebrand factor. All experiments were performed using platelets from patients 1 and 2, and no discernible differences were obtained. Only the results obtained with the platelets of patient 1 are presented in this study. The patient with Bernard-Soulier syndrome also had a long Ivy bleeding time (> 15 min) but had elevated levels of factor VIII complex (factor VIII activity 240%; von Willebrand antigen 305%; von Willebrand factor activity 210%) whereas ristocetin- and bovine von Willebrand factor-induced aggregation were absent. In vitro correction of ristocetin-induced aggregation of the PRP of the patients with von Willebrand's disease was achieved by addition of the plasma from the patient with Bernard-Soulier syndrome.[4] All patients demonstrated normal aggregation to ADP, collagen, adrenaline, and concanavalin A.

Iodination of washed platelets. Either 0.9 mCi ^{131}I or 1 mCi ^{125}I followed by 5 μl 0.1 M sodium phosphate, pH 7.4, containing 0.25 nmol of lactoperoxidase was added to stirred, washed 1-ml platelet suspensions containing 10^9 platelets (11). Five 5-μl aliquots of freshly prepared hydrogen peroxide solution (1 mM in 0.154 M sodium chloride) were then added at 10-s intervals. 3 ml of a buffer containing 0.154 M sodium chloride, 0.01 M Tris, and 1 mM EDTA, pH 7.4, was added. The platelets were sedimented by centrifugation, washed twice with 4 ml of the same buffer, and solubilized in 2–3% SDS as described above.

Iodination of chymotrypsin. 10 mg chymotrypsin in 2 ml 0.134 M sodium chloride, 0.01 M sodium phosphate, pH 7.4 was treated with 0.2 mCi ^{125}I followed by 5 μl 0.1 M sodium phosphate, pH 7.4, containing 0.25 nmol of lactoperoxidase with stirring. Five 5-μl aliquots of freshly prepared hydrogen peroxide solution (0.01 M in 0.154 M sodium chloride) were then added at 10-s intervals. The mixture was dialyzed against four changes of 0.134 M sodium chloride, 0.01 M sodium phosphate, pH 7.35 to remove excess hydrogen peroxide and noncovalently bound iodide. The labeled chymotrypsin had a specific activity of 1.2 μCi/mg protein.

Binding studies of ^{125}I-chymotrypsin to washed normal platelets. 1-ml washed normal platelets (10^9 platelets), were incubated at 37°C with 50 μg ^{125}I-labeled chymotrypsin for 15 min. The platelets were sedimented by centrifugation, washed three times using 0.154 M sodium chloride, 0.01 M Tris, and 1 mM EDTA, pH 7.4, and the radioactivity associated with the platelets was determined.

Gel electrophoresis. Samples of SDS-solubilized platelets (10–20 μl containing 100 μg protein) were analyzed in a reduced state by treatment with 50 μl of a mixture containing 2% 2-mercaptoethanol, 8 M urea, and 2% SDS, and was 0.01 M in sodium phosphate, pH 7.35. After addition of the buffered solution, samples were immediately immersed in boiling water for 3 min and were electrophoresed on 5% acrylamide gels as previously described (11). The gels were 13 cm long and contained 0.1% SDS. After electrophoresis, gels were either frozen for slicing or stained for protein with Coomassie Brilliant Blue or for carbohydrate with periodic acid-Schiff's reagent. To see the glycoproteins on the periodic acid-Schiff's reagent-stained gels, it was necessary to use samples containing 800 μg protein (80–100 μl). The radioactive iodine distribution in gels was determined by slicing the frozen gels laterally into 1.6-mm sections and determining the radioactivity associated with each slice. For double labeling experiments, samples labeled with ^{125}I were mixed with those labeled with ^{131}I, treated as above with 2% 2-mercaptoethanol, and electrophoresed. The isotope distribution within the gel was the same for a given sample whether the sample was run individually or mixed with another. The distribution of label was expressed graphically with counts per slice/total number of counts per gel × 100 plotted on the ordinate and the slice number plotted on the abscissa.

Materials. ^{131}I and ^{125}I were purchased from the Commissariat à l'Energie Atomique, Saclay, France and were supplied as NaI and essentially carrier free. Chymotrypsin was from Worthington Biochemical Corp., Freehold, N. J. Ristocetin lot 3 was a gift from the Lundbeck Co. A/S, Copenhagen-Valby, Denmark, and ristocetin lot 3003-109-30 was a gift from the Abbott Laboratories, North Chicago, Ill. Ristocetin was stored at −70°C as a stock solution dissolved in 0.154 M sodium chloride containing 200 mg/ml. Dilutions of the stock solutions were made before use with 0.154 M sodium chloride. Lactoperoxidase was isolated according to Morrison and Hultquist (20). Apyrase (potato), DFP, and ADP were purchased from the Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade.

RESULTS

Action of different ristocetin preparations on platelet membrane glycoproteins. Four times washed normal platelets do not aggregate in the presence of ristocetin unless a source of von Willebrand factor is added. When platelets were incubated with different ristocetin preparations before the addition of normal plasma, different effects on the platelets were observed. Platelets preincubated with ristocetin lot 3003-109-30 aggregated normally upon the addition of plasma, while platelets preincubated with ristocetin lot 3, containing a protease contaminant, gave a reduced aggregation response. The decrease in response was found to be time dependent and was inhibited by pretreatment of this preparation of ristocetin with DFP. Thus, it appeared that the decreased

[3] Factor VIII complex consists of at least three entities: factor VIII clotting activity, von Willebrand antigen, and von Willebrand factor activity. Factor VIII clotting activity is estimated by the correction of the partial thromboplastin time of a severe hemophilia A plasma (factor VIII activity < 1%) in the presence of kaolin; von Willebrand antigen is measured by immunological assays using precipitating antisera; and von Willebrand factor activity is estimated by the correction of ristocetin-induced aggregation of four times washed normal platelets. Values given are as a percentage of the entity present in a standard pool plasma.

[4] Jenkins, C. S. P., D. Meyer, and M-J. Larrieu. Unpublished observations.

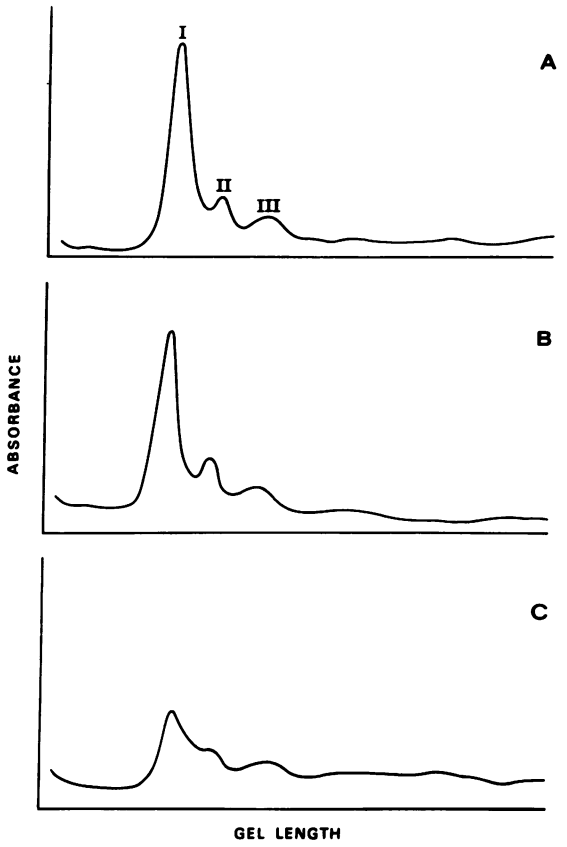


FIGURE 1 Action of different ristocetin preparations on platelet membrane glycoproteins. Platelets were washed according to Jenkins et al. (8): 1 ml of platelet suspension (10^9 platelets) was incubated with either 0.154 M sodium chloride (A), ristocetin lot 3003-109-30, 2 mg/ml (B), or ristocetin lot 3, 2 mg/ml (C) for 5 min at 37°C. The platelets were sedimented, solubilized in 2% SDS, treated with 2% 2-mercaptoethanol, immersed in boiling water for 3 min, and electrophoresed on 5% acrylamide gels containing 0.1% SDS. Developed gels were stained for carbohydrate and scanned. Glycoproteins I, II, and III are indicated.

response was due to the effect of a contaminant present in ristocetin lot 3 on the platelets.

To determine the possible action site of different ristocetin preparations, washed ristocetin-treated normal platelets were analyzed using SDS-disk gel electrophoresis after treatment with 2% 2-mercaptoethanol. Gels stained for protein did not reveal any discernible differences. Gels stained for carbohydrate demonstrated marked differences in the membrane glycoproteins. Platelets treated with 0.154 M sodium chloride or ristocetin lot 3003-109-30 (Fig. 1A and B) showed three major glycoprotein bands I, II, and III (following current notation) (11). However, analysis by SDS-acrylamide gel electrophoresis of platelets treated with ristocetin lot 3 showed that the glycoproteins were reduced in concentration with the greatest effect being on glycoprotein I (Fig. 1C). The intensity of staining and location of the major glycoproteins were not affected when ristocetin lot 3, which had been treated previously with DFP (0.05 M), was used.

Action of chymotrypsin on ristocetin-induced aggregation. Since the proteolytic enzyme contaminant present in ristocetin lot 3 was of unknown specificity, it was decided to examine the effect of a defined proteolytic enzyme.

Platelets incubated with various concentrations of chymotrypsin for 15 min at 37°C and thoroughly washed were tested for aggregation after addition of normal plasma and ristocetin lot 3003-109-30 (Fig. 2). Platelets that had been treated with chymotrypsin at 100 μg/ml failed to aggregate (Fig. 2D), while some aggregation occurred in platelets treated with chymotrypsin at 50 μg/ml (Fig. 2C). Chymotrypsin at 10 μg/ml was not found to affect the aggregation response under the experimental conditions employed (Fig. 2B).

Platelets incubated with chymotrypsin and washed were also analyzed by disk gel electrophoresis after treatment with 2% 2-mercaptoethanol. When stained for protein, these gels (not shown) revealed only slight differences from the control while those stained for car-

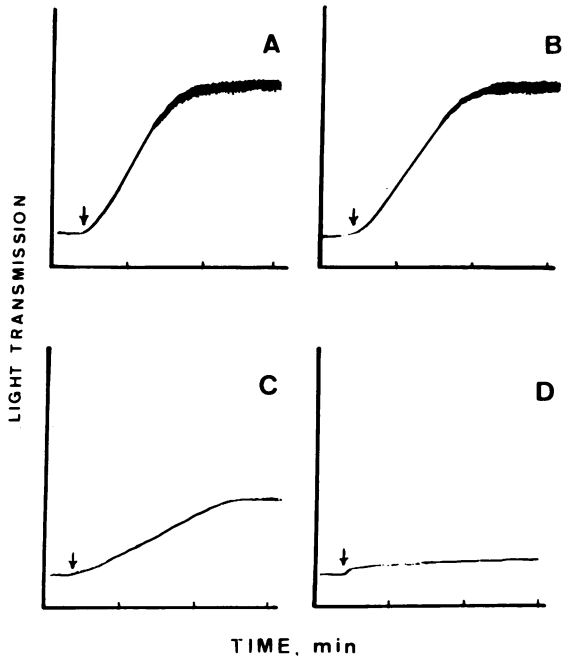


FIGURE 2 Action of chymotrypsin on ristocetin-induced aggregation. Platelets were washed as described in Fig. 1 and treated with either 0.134 M sodium chloride 0.01 M sodium phosphate pH 7.35 (A), or chymotrypsin 10 μg/ml (B), 50 μg/ml (C), or 100 μg/ml (D) in the same buffer. The platelets were washed three times and tested for aggregation with ristocetin lot 3003-109-30, 2 mg/ml, and standard pool plasma.

bohydrate (Fig. 3) showed that increasing amounts of chymotrypsin reduced the concentrations of the three major glycoproteins of the platelet membrane. Platelets

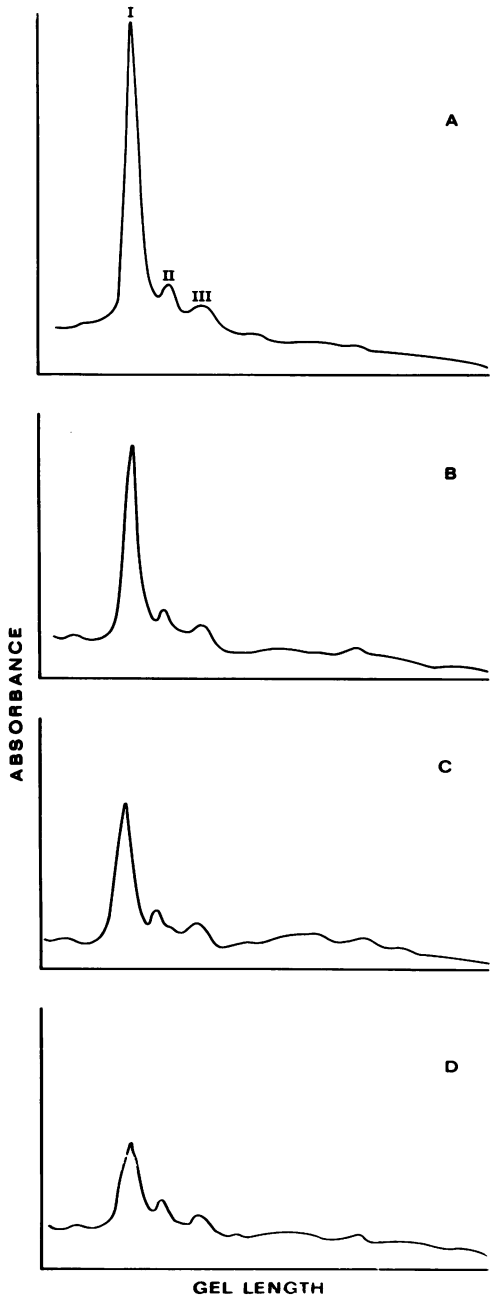


FIGURE 3 Action of chymotrypsin on platelet glycoproteins. Platelets were treated with buffer (A), or chymotrypsin at 10 μg/ml (B), 50 μg/ml (C), or 100 μg/ml (D), as described in Fig. 2, and washed three times. The platelet pellets were solubilized in 2% SDS, treated with 2% 2-mercaptoethanol, immersed in boiling water for 3 min, electrophoresed on 5% acrylamide gels, stained for carbohydrate, and scanned in a microdensitometer.

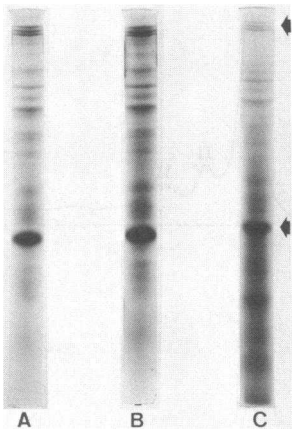


FIGURE 4 Polypeptide composition of normal platelets compared with that of platelets from patients with von Willebrand's disease and Bernard-Soulier syndrome. Washed platelets were solubilized in 2–3% SDS, treated with 2% 2-mercaptoethanol, immersed in boiling water for 3 min, electrophoresed on 5% acrylamide gels, and stained for protein. Gels A (normal) and B (von Willebrand's disease) contained 100 μg protein while gel C (Bernard-Soulier syndrome) contained 180 μg protein. Top arrow indicates myosin band. Bottom indicates actin band.

similarly treated with ^{125}I-chymotrypsin (50 μg/ml), after incubation and three washing steps, revealed that 0.1% of the total radioactivity added was associated with the platelets (0.055 μg ^{125}I-chymotrypsin/10^9 platelets).

Surface structure of platelets from patients with Bernard-Soulier syndrome and von Willebrand's disease. Since a reduced response to ristocetin occurs when the surface glycoproteins of normal platelets are altered, it was decided to investigate the surface structure of platelets from patients with Bernard-Soulier syndrome and von Willebrand's disease. To compare platelet-specific polypeptides and glycoproteins of washed normal platelets and washed platelets from patients with Bernard-Soulier syndrome and von Willebrand's disease, solubilized reduced platelets were analyzed by SDS-acrylamide disk gel electrophoresis, and the gels were stained for protein and carbohydrate. To determine whether protein exposed on the membrane surface of platelets from these disorders differs from the normal, such platelets were labeled with radioactive iodine by the lactoperoxidase iodination technique and were compared with platelets from normal donors.

Analysis of platelets from patients with Bernard-Soulier syndrome and von Willebrand's disease by gel electrophoresis. Solubilized platelets from normal donors (3.4 mg protein/10^9 platelets) and from patients with Bernard-Soulier syndrome (6.5 mg protein/10^9 platelets) and von Willebrand's disease (4 mg protein/10^9 platelets) were treated with 2% 2-mercaptoethanol,

analyzed by SDS-acrylamide gel electrophoresis, and the gels were stained for protein (Fig. 4) and carbohydrate (Fig. 5). The proteins of solubilized normal platelets and platelets from patients with von Willebrand's disease were discernible using 100 μg protein while 180 μg was necessary to see those of the Bernard-Soulier platelets. Bernard-Soulier platelets showed numerous low molecular weight polypeptides which were not present in either normal platelets or platelets from the patients with von Willebrand's disease. In addition, the platelet myosin and actin bands obtained from Bernard-Soulier platelets were found to be less intense than in the normal.

Periodic acid-Schiff's reagent staining of gels of solubilized normal platelets or platelets from the patients with von Willebrand's disease demonstrated three glycoprotein bands (I, II, and III) using 800 μg protein. When the solubilized platelets from the patient with Bernard-Soulier syndrome were analyzed at this concentration, no bands were observed: at 1,100 μg protein, one diffuse band in the glycoprotein I region was seen. Even at 1,500 μg protein, glycoproteins II and III were not detected.

These results indicate that there is an extensive change in the surface of platelets from the patient with Bernard-Soulier syndrome, while the platelets from the patients with von Willebrand's disease appear to be the same as normal.

Lactoperoxidase-catalyzed iodination of platelets from patients with Bernard-Soulier syndrome and von Willebrand's disease. Platelets from six normal donors were labeled with either ^{131}I or ^{125}I. Fig. 6 shows the iodination pattern obtained when ^{131}I-labeled platelets from one of these donors were solubilized, mixed with solubilized ^{125}I-labeled platelets from another donor, treated with 2% 2-mercaptoethanol, immersed in boiling water for 3 min, and electrophoresed on the same gel. Four major iodinated components were observed which lay in the glycoprotein region. Comparison with gels which had been stained for carbohydrate revealed that three of these iodinated species coincided with the three major glycoprotein bands I, II, and III (11), while the fourth ran at a slightly higher molecular weight than II and stained, albeit weakly, for carbohydrate. It is proposed that this new component which is weakly stained by periodic acid-Schiff's reagent be termed IIa and that the former II be termed IIb. In addition to these four components, other uncharacterized polypeptides of lower molecular weights were also iodinated. When samples from other normal donors were compared, no discernible differences were observed in the amounts of the glycoproteins and polypeptides that were labeled. The ratios of I : IIa : IIb : III from the six normal donors were 1 : 1 : 1 : 3.

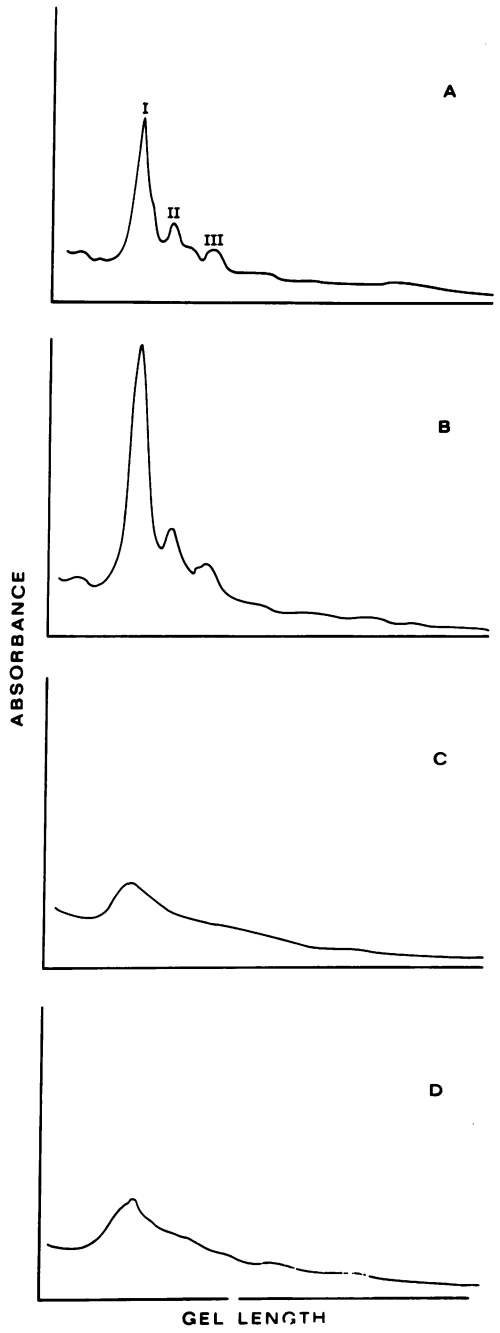


FIGURE 5 Glycoprotein composition of normal platelets compared with that of platelets from patients with von Willebrand's disease and Bernard-Soulier syndrome. Washed platelets were solubilized in 2–3% SDS, treated with 2% 2-mercaptoethanol, immersed in boiling water for 3 min, electrophoresed on 5% acrylamide gels, stained for carbohydrate, and scanned in a microdensitometer. Tracings A (normal) and B (von Willebrand's disease) were of gels which contained 800 μg protein and tracings C and D (Bernard-Soulier syndrome) of gels containing 1,100 and 1,500 μg protein, respectively.

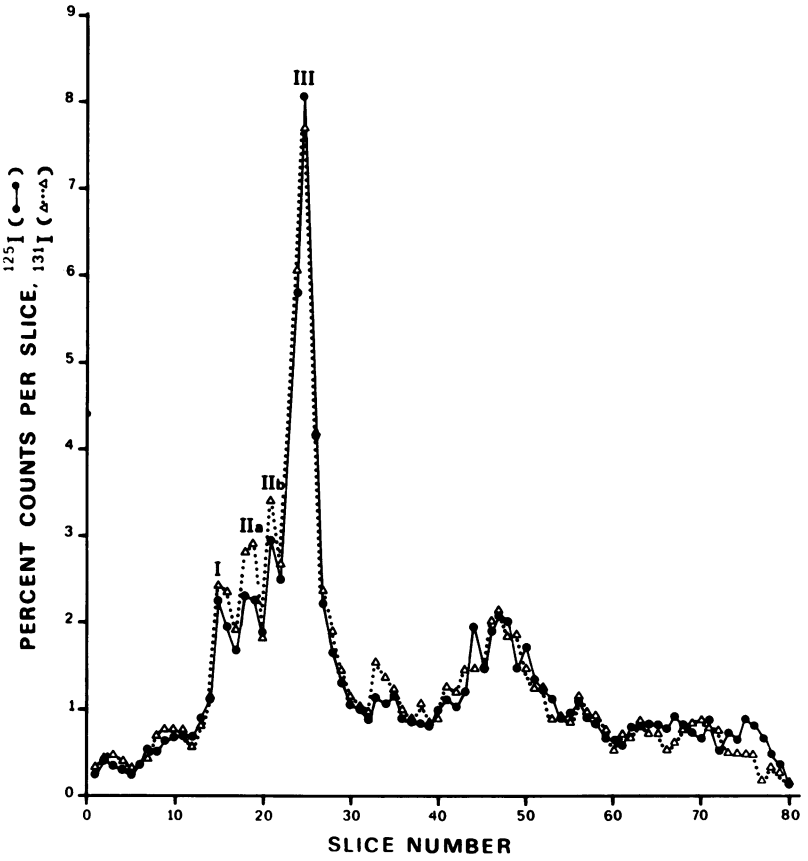


FIGURE 6 The exposed polypeptides and glycoproteins of the surface membranes of normal platelets using reduced samples. Platelets from normal donors were washed according to Massini and Lüscher (14). Platelet suspensions were adjusted to 10^9 platelets/ml. 1-ml aliquots of normal platelets were labeled with either ^{131}I or ^{125}I using lactoperoxidase (11). Labeled platelets were washed twice and solubilized in 2–3% SDS. Solubilized samples from different normal donors labeled with ^{131}I or ^{125}I were mixed in such amounts that spillover of ^{131}I into the ^{125}I channel on the gamma spectrometer would not be significant. The mixed sample was treated with 2% 2-mercaptoethanol, immersed in boiling water for 3 min, electrophoresed on 5% acrylamide gels, sliced laterally into 1.6-mm sections, and the radioactivity determined. The percentage total counts in each slice was calculated according to the following equation: counts per slice per total counts on gel × 100.

Suspensions of Bernard-Soulier platelets, isolated either by differential centrifugation or by the ADP method, were labeled with ^{125}I using lactoperoxidase. Labeled platelets isolated by differential centrifugation were solubilized and mixed with solubilized ^{131}I-labeled normal platelets isolated by the same technique, treated with 2% 2-mercaptoethanol, and electrophoresed on the same gel. Fig. 7A shows the distribution of these isotopes in the gel. The patient's platelets, which had been isolated by the ADP method and labeled with ^{125}I, were solubilized, mixed with solubilized ^{131}I-labeled normal platelets isolated by the same technique, treated with 2% 2-mercaptoethanol, and electrophoresed on the same gel (Fig. 7B). It should be noted that both the patient's iodination patterns as well as those of normal donors were not influenced by the methods of platelet isolation employed. It has been shown (21) that platelets are iodinated at a faster rate than lymphocytes. Since the iodination pattern of Bernard-Soulier platelets isolated by differential centrifugation (Fig. 7A), which contained 15% white cell contamination, was not essentially different from that of Bernard-Soulier platelets isolated by the ADP method (Fig. 7B) containing only 1% white cell contamination, it would therefore seem that white cells do not contribute significantly to the iodination patterns observed.

The labeling pattern observed with the platelets of the patient revealed marked differences compared with normal platelets. Most notable differences were the presence of numerous uncharacterized polypeptides of lower mo-

lecular weight absent on normal platelets and the relative decreased iodination in the glycoprotein region. In addition, glycoproteins I and IIb were not apparent on the Bernard-Soulier platelet.

The platelets from the patients with von Willebrand's disease, labeled with ^{125}I, were solubilized, and mixed with a representative sample of ^{131}I-labeled solubilized normal platelets. The mixtures were treated with 2% 2-mercaptoethanol, and electrophoresed on the same gel. The ratios of the iodinated components of both the platelets from the patients and of normal donors were found to be similar (Fig. 8), indicating that the same relative concentrations of glycoproteins and polypeptides were exposed on both membrane surfaces.

The data obtained from the iodination experiments reveal that there is a decrease in the iodination of the membrane glycoproteins of the patient's platelets with Bernard-Soulier syndrome relative to other membrane constituents, while the exposed polypeptides and glycoproteins of the platelets from the patients with von Willebrand's disease were iodinated as in normals.

DISCUSSION

There is increasing evidence that plasma membrane glycoproteins play a significant role in the platelet response to a variety of stimuli. It has been argued that glycoprotein II is the proteolytic site of thrombin (22). Platelets from patients with Glanzmann's thrombasthenia, which are not aggregated by ADP, have been shown to have abnormalities in the relative amounts of membrane glycoproteins (23). Majerus and Brodie (24) have shown that the lectins from *Phaseolus vulgaris* stimulate the release reaction of platelets by interaction with carbohydrate residues on the membrane. Greenberg and Jamieson (25) showed that other lectins were able to induce the platelet-release reaction and in turn aggregation.

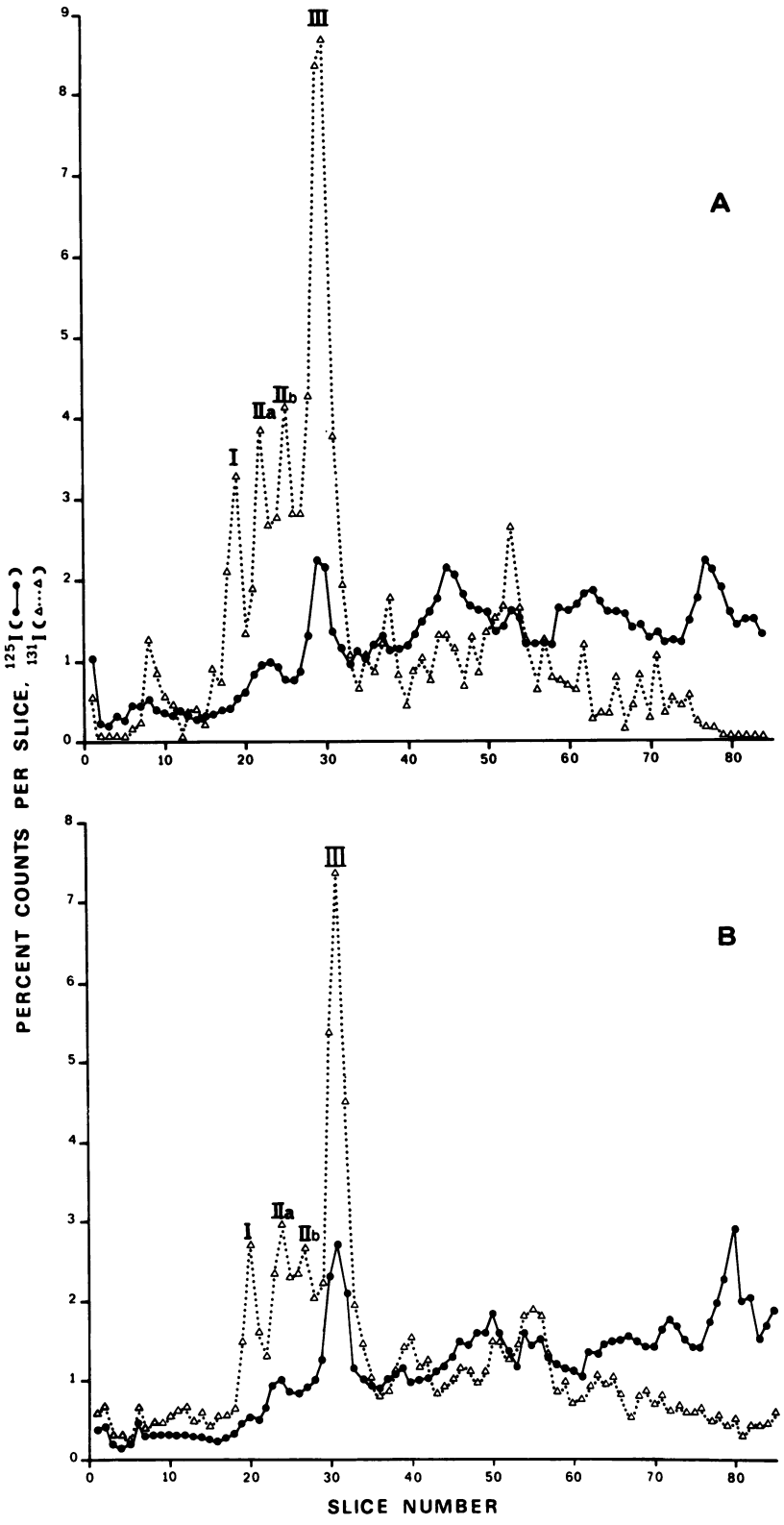
The studies presented in this paper were designed in an attempt to identify the receptor on the platelet plasma membrane involved in ristocetin-induced aggregation. Two approaches were chosen. The first involved investigations on normal platelets rendered unresponsive to ristocetin in the presence of normal plasma and the second involved disorders where ristocetin-induced aggregation is absent. Earlier work [1] had shown that platelets incubated with ristocetin lot 3 have a decreased aggregation response on addition of normal plasma. This suggested that a surface component(s) of the platelet was altered by this preparation. This effect is due to a proteolytic contaminant in the preparation which is active in this respect. The enzyme responsible has not been characterized.

The components affected on the platelet surface by this protease include glycoproteins I and II. A ristocetin preparation without a proteolytic contaminant (lot 3003-109-30), even after prolonged incubation with platelets, is without effect on the platelets' responsiveness.

The other proteolytic enzyme investigated was chymotrypsin. Trypsin (11) and chymotrypsin both cleave platelet membrane glycoproteins, but chymotrypsin, in contrast to trypsin does not induce the platelet-release reaction (26). Therefore, the loss of platelet aggregation together with the reduced concentration of glycoproteins I, II, and III is not related to the loss or the action of platelet-released products but rather to the direct action of chymotrypsin on the platelet. The low amount of ^{125}I-chymotrypsin bound to the platelet under the experimental conditions used demonstrates that chymotrypsin is not retained on the membrane surface; it therefore seems unlikely that residual enzyme inhibits aggregation by interaction with the aggregating agent(s). Chymotrypsin cleaves the three major glycoproteins while the proteolytic contaminant present in ristocetin lot 3 cleaves only the two with the highest molecular weights. These data raise the possibility that a glycoprotein may be involved in the receptor engaged in ristocetin-induced aggregation.

Acrylamide gel electrophoresis of samples followed by staining of protein and carbohydrate, allows estimation of the individual polypeptides and glycoproteins while lactoperoxidase-catalyzed iodination permits the investigation of those components which are exposed on the membrane surface. Gel electrophoresis in SDS separates the platelets' proteins into at least 25 distinct bands and the three previously mentioned glycoproteins (27, 28). Iodination studies showed that these glycoproteins are exposed on the platelet membrane surface (11). In the present study, the reduction of iodinated, solubilized, normal platelets, if carried out under the conditions employed (2% 2-mercaptoethanol, followed by immersing the samples in boiling water for 3 min), revealed an additional labeled component, which stained poorly for carbohydrate. It appears that IIa and IIb are separate components since platelets from patients with Glanzmann's thrombasthenia have normal amounts of IIa but only 40% of the normal concentration of IIb (29). The iodination patterns obtained from normal samples reduced in this way consistently showed the presence of these four labeled membrane components in the ratios 1:1:1:3. Samples which were nonreduced or reduced with dithiothreitol do not allow distinction between these four glycoproteins (29). Reduction of samples with 2% 2-mercaptoethanol would appear useful since it allows the recognition of more iodinated protein entities of different molecular weights.

The second approach in these studies involved investigation of disorders where platelet response to ristocetin is abnormal. The abnormality associated with von


A
B
I
IIa
IIb
III
PERCENT COUNTS PER SLICE, ^{125}I (●—●), ^{131}I (△···△)
SLICE NUMBER

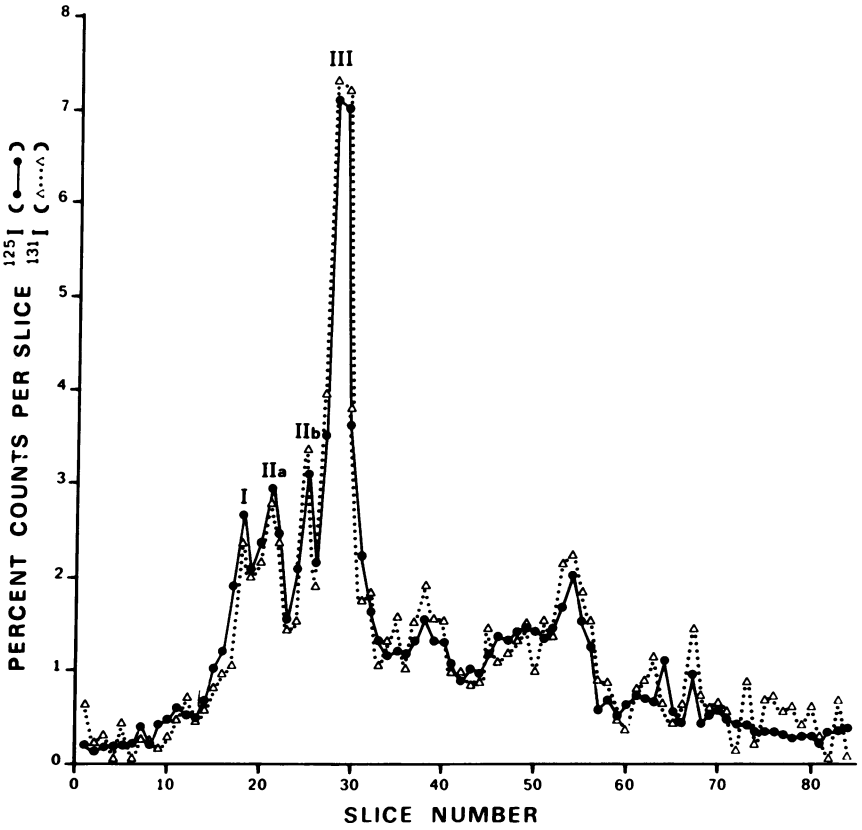


FIGURE 8 Comparison of the exposed polypeptides and glycoproteins of the surface membranes of normal platelets and platelets from a patient with von Willebrand's disease using reduced samples. Platelets from normal donors and a patient with von Willebrand's disease were washed according to Massini and Lüscher (14). Platelet suspensions were adjusted to 10^9 platelets/ml. Normal platelets were labeled with ^{131}I while those of the patient were labeled with ^{125}I. Samples of solubilized normal and patient's platelets were mixed and treated as described in Fig. 7.

Willebrand's disease is plasma in origin and the absence of aggregation induced by ristocetin is corrected by the addition of low concentrations of plasma (1-3). In addition, platelets from patients with von Willebrand's disease respond normally to other inducers of platelet aggregation (30) including bovine von Willebrand factor (17). Therefore, it would seem probable that platelets from this disorder would have the normal expression of membrane surface glycoproteins and polypeptides, and indeed, analysis of the iodinated platelets from patients with von Willebrand's disease revealed the normal expression of surface-labeled components, with the glycoproteins labeled in the ratios 1 : 1 : 1 : 3. Gel electrophoresis of the patient's platelets followed by staining for protein and carbohydrate gave the same results as those obtained with normal platelets.

In contrast to von Willebrand's disease the abnormal ristocetin response of Bernard-Soulier platelets is of

FIGURE 7 Comparison of the exposed polypeptides and glycoproteins of the surface membranes of normal platelets and platelets from a patient with Bernard-Soulier syndrome using reduced samples. Platelets from both normal donors and from the patient were washed by the method of Massini and Lüscher (14). Platelets were also isolated by the ADP method of Mohammad et al. (15). Normal platelets (10^9/ml) were labeled with ^{131}I while those of the patient (10^9/ml) were labeled with ^{125}I. Samples of solubilized normal platelets and solubilized platelets from the patient isolated by the same method were mixed, treated with 2% 2-mercaptoethanol, immersed in boiling water for 3 min, electrophoresed on 5% acrylamide gels, sliced laterally into 1.6-mm sections and the radioactivity determined. Tracing A: ^{125}I-labeled platelets from the patient and ^{131}I-labeled normal platelets (platelets washed according to Massini and Lüscher). Tracing B: ^{125}I labeled platelets from the patient and ^{131}I-labeled normal platelets (platelets isolated according to Mohammad et al. [15]).

platelet origin (5, 6) since the plasma of these patients contains normal levels of factor VIII complex and corrects ristocetin-induced aggregation in von Willebrand's disease. Bernard-Soulier platelets have decreased amounts of sialic acid and demonstrate reduced electrophoretic mobility (31). In addition, in Bernard-Soulier syndrome collagen-induced coagulant activity is absent, affinity for factors V and VIII is decreased, and factor XI is not detectable in washed platelet suspensions (32). Walsh et al. (32) have suggested that the membrane defect is relatively selective since the storage and release mechanisms for adenine nucleotides, heparin-neutralizing activity, and serotonin uptake are normal. They propose that the deficiency of sialic acid in the membranes of Bernard-Soulier platelets could account for the absence of bovine von Willebrand factor-induced aggregation and the deficient binding of factors V, VIII, and XI, while Gröttum and Solum (31) suggest that this may also explain the reduced electrophoretic mobility. Investigators have proposed that Bernard-Soulier platelets lack a membrane component present on normal platelets which interacts with von Willebrand factor and/or ristocetin (5, 6). Since a definite membrane defect most likely exists in Bernard-Soulier membranes, we have investigated the expression of membrane-surface glycoproteins and polypeptides in an attempt to locate the defect(s) at the molecular level, and to compare any differences with those found with normal platelets rendered unresponsive by proteolytic action.

Gel electrophoresis of the patient's platelets followed by staining for protein and carbohydrate revealed a decrease in the density of staining of platelet-specific proteins and glycoproteins, with the presence of numerous low molecular weight polypeptides absent on normal platelets. While 800 μg protein was sufficient to detect the three major glycoproteins of normal platelets, 1,500 μg protein of Bernard-Soulier platelets only resulted in the distinction of one diffuse band in the glycoprotein I region. In addition, iodination studies revealed numerous uncharacterized polypeptides of lower molecular weight exposed on the surface of Bernard-Soulier platelets and, relative to total incorporated radioactive iodine, a decrease in the iodination of the glycoprotein region. These molecular abnormalities associated with the membrane of Bernard-Soulier platelets are consistent with the findings that such platelets contain less sialic acid and demonstrate a decreased electrophoretic mobility (31).

Glycoproteins on normal platelets label in the ratios 1 : 1 : 1 : 3 when analyzed after reduction with 2% 2-mercaptoethanol. Analysis of the radioactive distribution in the gels from labeled Bernard-Soulier platelets showed that the relative amounts of label in glycoproteins I, IIa, IIb, and III were different from normal, with a marked reduction in the iodination of glycoproteins IIa and III. The abnormal pattern observed with Bernard-Soulier platelets could be due to the absence or alteration of some of these components. It is not possible to distinguish between these two alternatives at present, but the data indicate that an abnormality exists in the membrane glycoproteins of the platelets of this patient with Bernard-Soulier syndrome and that there are on the membrane surface numerous uncharacterized lower molecular weight polypeptides not present on normal platelets. The qualitative pattern of protein staining, periodic acid-Schiff's reagent staining, and lactoperoxidase labeling all indicate an extensive change in the cell surface. There is a qualitatively very significant alteration in the Bernard-Soulier platelets which is not seen in platelets from patients with von Willebrand's disease.

It is not known if these abnormalities exist in the platelets of other patients with Bernard-Soulier syndrome since we have not been able to study any others due to the rarity of this disorder. However, four iodinations of these platelets were performed on two separate occasions and the same results were obtained on each study.

In addition to the absence of ristocetin-induced aggregation in Bernard-Soulier syndrome, various other functional abnormalities have been reported (5, 6, 32). It is therefore difficult to correlate the membrane abnormalities to a particular function. In the present study a loss of ristocetin-induced aggregation occurs concurrently with the loss of normal platelet membrane glycoproteins. It is tempting to suggest that membrane glycoproteins play a functional role in ristocetin-induced aggregation, however other possibilities may not be excluded. Iodination reflects not only the exposure of platelet membrane proteins but also their ability to be iodinated and is therefore not a means of investigating the concentration of proteins in the membrane. It is possible that the receptor involved in ristocetin-induced aggregation on normal platelets is not labeled or it may be present in such low concentrations that it is not detectable by iodination. The receptor could also be one of the iodinated components of lower molecular weight than the glycoproteins and its absence would not be observed in Bernard-Soulier syndrome due to the large number of additional iodinated polypeptides present. Bernard-Soulier platelets may not lack the receptor but could have the same number of receptor sites per platelet as do normal platelets but with a reduced density on the membrane surface owing to the increased surface area of the abnormal platelet. Allain et al. (33) showed that platelets fixed with paraformaldehyde aggregate to ristocetin. This might indicate that lateral mobility of membrane receptors is not a prerequisite for this aggregation as it is for lectin-mediated aggregation of

other cell types (34, 35). Thus reduced density of receptor sites on the platelets of the patient could have a pronounced effect on ristocetin-induced aggregation.

Addendum. Since this paper was submitted, Nurden and Caen have published (*Nature (Lond.)*. 1975. **255**: 720–722.) studies on the platelets of two patients with Bernard-Soulier syndrome and have shown a decrease in staining of glycoprotein I relative to other membrane components.

ACKNOWLEDGMENTS

We would like to thank the Lundbeck and Abbott Laboratories for kindly supplying ristocetin, and Professor D. Alagille for allowing us to study the patient with Bernard-Soulier syndrome.

This work was supported by grants from the Institute National de la Santé et de la Recherche Médicale, France, the Swiss National Science Foundation, Roche Research Foundation, and NIH (HL 15616).

REFERENCES

1. Howard, M. A., and B. G. Firkin. 1971. Ristocetin—a new tool in the investigation of platelet aggregation. *Thromb. Diath. Haemorrh.* **26**: 362–369.
2. Meyer, D., C. S. P. Jenkins, M. D. Dreyfus, and M-J. Larrieu. 1973. Experimental model for von Willebrand's disease. *Nature (Lond.)*. **243**: 293–294.
3. Weiss, H. J., J. Rogers, and H. Brand. 1973. Defective ristocetin-induced platelet aggregation in von Willebrand's disease and its correction by factor VIII. *J. Clin. Invest.* **52**: 2697–2707.
4. Tschopp, T. B., H. J. Weiss, and H. R. Baumgartner. 1974. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. *J. Lab. Clin. Med.* **83**: 296–300.
5. Howard, M. A., R. A. Hutton, and R. M. Hardisty. 1973. Hereditary giant platelet syndrome: a disorder of a new aspect of platelet function. *Br. Med. J.* **2**: 586–588.
6. Caen, J. P., and S. Levy-Toledano. 1973. Interaction between platelets and von Willebrand Factor provides a new scheme for primary haemostasis. *Nat. New Biol.* **244**: 149–150.
7. Weiss, H. J., T. B. Tschopp, H. R. Baumgartner, I. I. Sussman, M. M. Johnson, and J. J. Egan. 1974. Decreased adhesion of giant (Bernard-Soulier) platelets to subendothelium. Further implications on the role of the von Willebrand Factor in hemostasis. *Am. J. Med.* **57**: 920–925.
8. Jenkins, C. S. P., D. Meyer, M. D. Dreyfus, and M-J. Larrieu. 1974. Willebrand Factor and ristocetin. I. Mechanism of ristocetin-induced platelet aggregation. *Br. J. Haematol.* **28**: 561–578.
9. Morgan, F. J., A. Goh, and B. G. Firkin. 1974. Proteolytic activity in ristocetin preparations. *Thromb. Res.* **5**: 565–569.
10. Phillips, D. R., and M. Morrison. 1971. Exposed protein on the intact human erythrocyte. *Biochemistry.* **10**: 1766–1771.
11. Phillips, D. R. 1972. Effect of trypsin on the exposed polypeptides and glycoproteins in the human platelet membrane. *Biochemistry.* **11**: 4582–4588.
12. Huber, C. T., and M. Morrison. 1973. Heterogeneity of the outer membrane of mitochondria. *Biochemistry.* **12**: 4274–4282.
13. Aster, R. H., and J. H. Jandl. 1964. Platelet sequestration in man. I. Methods. *J. Clin. Invest.* **43**: 843–855.
14. Massini, P., and E. F. Lüscher. 1974. Some effects of ionophores for divalent cations on blood platelets. Comparison with the effects of thrombin. *Biochim. Biophys. Acta.* **372**: 109–121.
15. Mohammad, S. F., R. L. Reddick, and R. G. Mason. 1975. Characterization of human platelets separated from blood by ADP-induced aggregation. *Am. J. Pathol.* **79**: 81–94.
16. Born, G. V. R. 1962. Aggregation of blood platelets by adenosine disphosphate and its reversal. *Nature (Lond.)*. **194**: 927–929.
17. Meyer, D., C. S. P. Jenkins, M. D. Dreyfus, E. Fressinaud, and M-J. Larrieu. 1974. Willebrand Factor and ristocetin. II. Relationship between Willebrand Factor, Willebrand antigen and Factor VIII activity. *Br. J. Haematol.* **28**: 579–599.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
19. Alagille, D., F. Josso, J-L. Binet, and M. L. Blin. 1964. La dystrophie thrombocytaire hémorragipare. Discussion Nosologique. *Nouv. Rev. Fr. Hematol.* **4**: 755–790.
20. Morrison, M., and D. E. Hultquist. 1963. Lactoperoxidase. II. Isolation. *J. Biol. Chem.* **238**: 2847–2849.
21. Gates, R. E., D. R. Phillips, and M. Morrison. 1975. The distinguishing characteristics of the plasma membrane are its exposed proteins. *Biochem. J.* **147**: 373–376.
22. Phillips, D. R., and P. Poh Agin. 1974. Thrombin substrates and the proteolytic site of thrombin action on human-platelet plasma membranes. *Biochim. Biophys. Acta.* **352**: 218–227.
23. Nurden, A. T., and J. P. Caen. 1974. An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. *Br. J. Haematol.* **28**: 253–260.
24. Majerus, P. W., and G. N. Brodie. 1972. The binding of phytohemagglutinins to human platelet plasma membranes. *J. Biol. Chem.* **247**: 4253–4257.
25. Greenberg, J. H., and G. A. Jamieson. 1974. The effect of various lectins on platelet aggregation and release. *Biochim. Biophys. Acta.* **345**: 231–242.
26. Davey, M. G., and E. F. Lüscher. 1967. Actions of thrombin and other coagulant and proteolytic enzymes on blood platelets. *Nature (Lond.)*. **216**: 857–858.
27. Nachman, R. L., and B. Ferris. 1972. Studies on the proteins of human platelet membranes. *J. Biol. Chem.* **247**: 4468–4475.
28. Phillips, D. R., and P. Poh Agin. 1973. Thrombin-induced alterations in the surface structure of the human platelet plasma membrane. *Ser. Haematol.* **6**: 292–310.
29. Phillips, D. R., C. S. P. Jenkins, E. F. Lüscher, and M-J. Larrieu. 1975. Molecular differences of exposed surface proteins on thrombasthenic platelet plasma membranes. *Nature (Lond.)*. **257**: 599–600.
30. Weiss, H. J. 1968. Von Willebrand's disease—diagnostic criteria. *Blood.* **32**: 668–679.
31. Gröttum, K. A., and N. O. Solum. 1969. Congenital thrombocytopenia with giant platelets: a defect in the platelet membrane. *Br. J. Haematol.* **16**: 277–290.
32. Walsh, P. N., D. C. B. Mills, F. I. Pareti, G. J. Stewart, D. E. Macfarlane, M. M. Johnson, and J. J. Egan. 1975. Hereditary giant platelet syndrome. Absence of collagen-

induced coagulant activity and deficiency of Factor-XI binding to platelets. *Br. J. Haematol.* **29**: 639–655.
33. Allain, J.-P., H. A. Cooper, R. H. Wagner, and K. M. Brinkhous. 1975. Platelets fixed with paraformaldehyde: a new reagent for assay of von Willebrand Factor and platelet aggregating factor. *J. Lab. Clin. Med.* **85**: 318–328.
34. Rosenblith, J. Z., T. E. Ukena, H. H. Yin, R. D. Berlin, and M. J. Karnovsky. 1973. A comparative evaluation of the distribution of concanavalin A-binding sites on the surfaces of normal, virally-transformed, and protase-treated fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 1625–1629.
35. Nicolson, G. L. 1973. Temperature-dependent mobility of concanavalin A sites on tumour cell surfaces. *Nat. New Biol.* **243**: 218–220.