Analysis of the Glycoprotein and Protein Composition of Bernard-Soulier Platelets by Single and Two-dimensional Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

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ABSTRACT Previous reports have described conflicting results concerning the glycoprotein (GP) and protein composition of Bernard-Soulier platelets. In view of this controversy we have analyzed the platelets of four Bernard-Soulier patients using improved single and two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis procedures. An absence of staining for carbohydrate of membrane GP Ib was characteristic for the platelets of each patient. Major periodate-Schiff staining bands corresponding to membrane GP IIb, IIIa, and IIIb were clearly detected and their presence was confirmed by two-dimensional SDS-polyacrylamide gel electrophoresis. The protein content of the Bernard-Soulier platelets was increased two- to fourfold. However, analysis of their protein composition using 7–12% acrylamide gradient gels showed normal polypeptide profiles. Lactoperoxidase-catalyzed ^{125}I-labeling of the Bernard-Soulier platelet surface proteins was followed by SDS-polyacrylamide gel electrophoresis and autoradiography. No labeling in the Ib position was detected whereas the other major membrane GP, including Ia and IIa, were normally located. In contrast, GP Ib was clearly detected by periodate-Schiff staining and autoradiography when normal human platelets that had been exhaustively treated with neuraminidase before the lactoperoxidase-catalyzed iodination were analysed. No abnormalities were detected in the GP patterns of membranes isolated from the patients' erythrocytes. Only a severe molecular abnormality or possible deletion of GP Ib could account for this major platelet lesion in the Bernard-Soulier syndrome.

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Received for publication 23 October 1980 and in revised form 29 December 1980.

INTRODUCTION

The Bernard-Soulier (B-S)[1] syndrome is a congenital platelet disorder with an autosomal recessive inheritance that was originally characterised by the association of a prolonged bleeding time, the presence of abnormally large platelets on peripheral blood smears, an often observed thrombocytopenia, and a defective prothrombin consumption test (1). Subsequent studies have suggested that the bleeding tendency arises from an abnormality in the platelet-vessel wall interaction, or more specifically, in a diminished platelet ability to adhere to subendothelium (2–4). B-S platelets are not agglutinated by bovine factor VIII (5, 6) or by ristocetin in the presence of normal human plasma (7, 8). A diminished binding of certain coagulation factors to B-S platelets has been noted (6) whereas a reduced binding of ^{125}I-labeled thrombin to B-S platelets was correlated with a decreased aggregation response specifically observed with this stimulus (9, 10). Finally, B-S platelets have been shown to lack the receptor for quinine- or quinidine-dependent antibodies (11).

Gröttum and Solum (12) first reported that the platelets of two B-S patients had a reduced sialic acid content and electrophoretic mobility. This observation suggested the presence of a membrane glycoprotein abnormality. Nurden and Caen (13) subsequently located a severe reduction in the periodate-Schiff (PAS)-staining intensity of the then-termed glycoprotein (GP) I band following the analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of membranes isolated from the platelets of two B-S patients. This abnormality was confirmed by the direct

[1] *Abbreviations used in this paper*: B-S syndrome, Bernard-Soulier syndrome; GP, glycoprotein; PAS reaction, periodate-Schiff reaction; PRP, platelet-rich plasma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

analysis of unfractionated B-S platelets by SDS-PAGE (3). In contrast, Jenkins et al. (14) reported other findings after their studies on the platelets of a different B-S patient. These authors detected a diffuse PAS band in the GP I position after SDS-PAGE and were unable to detect PAS-staining in the GP II and III positions. Furthermore, widespread changes in both the platelet protein composition and the ^{125}I-labeling pattern of the surface-orientated polypeptides were observed and emphasized. In recently published studies, Hagen et al. (15), and Shulman and Karpatkin (16) analyzed B-S platelets by crossed immunoelectrophoresis using rabbit antisera raised against normal human platelets (15) or isolated platelet membranes (16). Markedly different findings were reported. Hagen et al. (15) located a specific defect involving GP Ib and glycocalicin, a high molecular weight glycopeptide reported to be released from Ib as a result of Ca^{2+}-dependent protease activity (17). In contrast, Shulman and Karpatkin (16) noted numerous differences and suggested that the abnormalities observed in the B-S syndrome and those of a second hereditary platelet disorder, Glanzmann's thrombasthenia, reflected differences in endogenous membrane proteolysis.

In view of the apparent contradiction between our initial observations and those of Jenkins et al. (14), the increased complexity of the human platelet membrane GP composition as revealed by the introduction of improved SDS-PAGE procedures, and the lack of agreement in the reports on the analysis of B-S platelets by crossed immunoelectrophoresis, we now describe further studies performed on the platelets of four B-S patients. Analysis of the GP and proteins of ^{125}I-labeled platelets of each patient by refined single and two-dimensional SDS-PAGE procedures has established that B-S platelets possess a specific and characteristic membrane GP lesion. Furthermore, a comparison with the results obtained for control platelets labeled with ^{125}I after treatment with neuraminidase to remove exposed sialic acid residues has shown that a decreased sialic acid content cannot alone account for the observed B-S platelet surface alterations.

METHODS

Materials

Dextran T 500 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and sodium metrizoate solution (32.8% wt/vol, density 1.20 g/ml) from Nyegaard & Co., Oslo, Norway. All materials for SDS-PAGE were of electrophoresis purity grade and were obtained from Bio-Rad Laboratories, Richmond, Calif. High and low molecular weight protein standards used for molecular weight determinations following SDS-PAGE were also obtained from Bio-Rad Laboratories. Schiff's reagent was prepared from Fuchsine Diament (Reactifs RAL, purchased from the Société Chimique Pointet Girard, Clichy, France). Neuraminidase (Vibrio cholera 500 U/ml) was purchased from Behringwerke, Marburg/Lahn, West Germany, Carrier-free Na^{125}I (1 mCi/10 μl, in NaOH solution, pH 8–11) was obtained from the Radiochemical Center, Amersham, England. Lactoperoxidase was obtained from Sigma Chemical Co., St. Louis, Mo. Purified orosomucoid was a gift from Dr. Graham Jamieson (American National Red Cross Blood Research Laboratory, Bethesda, Md.) Autoradiography was performed using Kodak X-Omat MA film.

Subjects

Four patients with platelet function defects typical of the B-S syndrome have been studied. N.V. is an adult woman previously studied by us (3); R.B. is an adult man whose case history was reported by Bernard et al. (18); W.B. is an adult woman studied previously by Devaraj et al. (19) and examined by us through the courtesy of Dr. Frank Hill (The Children's Hospital, Birmingham, England); D.S. was an 11-yr-old boy at the time of study and together with N.V. and R.B. formed part of the report by Rendu et al. (20). Normal human blood was obtained from healthy adult volunteers. Informed consent was given and all studies were performed according to the principals of the Declaration of Helsinki.

Platelet isolation

Blood (9 vol) was taken by venipuncture directly into an acid-citrate-dextrose anticoagulant (1 vol) as described (3). Platelet-rich plasma (PRP) was prepared in either of two ways: (*a*) The blood was centrifuged at 120 *g* for 15 min at 15°C. This procedure was used for experiments with control blood only. (*b*) For the B-S patients and their appropriate controls, samples (2 ml) of blood were layered onto 5-ml vol of a solution of sodium metrizoate and dextran T 500 as described by Solum et al. (21). The erythrocytes were progressively clumped by the dextran and sedimented. The PRP was removed and the platelet and leukocyte content enumerated by phase contrast microscopy. The PRP was then centrifuged at 250 *g* for 5 min and the platelet and leukocyte content of the suspension re-evaluated. The sedimented cells were discarded. This process was repeated until the leukocyte contamination was <1% platelets (normally one or two centrifugations were sufficient).

The platelets in the PRP were finally sedimented by centrifugation at 3,000 *g* for 15 min and resuspended in small volumes (10 ml buffer for the platelets sedimented from 50 ml PRP) of 0.01 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, 5 mM glucose, and 1 mM EDTA. The concentrated platelet suspension was centrifuged at 2,600 *g* for 15 min in transparent plastic conical tubes (12 ml capacity). Any remaining erythrocytes settled to the base of the cone; the overlying platelets in the pellet were carefully resuspended and washed twice with half the original PRP volume of the above buffer.

Lactoperoxidase-catalyzed iodination

Washed control platelets were resuspended at 1×10^9 platelets/ml in 0.01 M Tris-HCl, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA. The platelets of the B-S patients were likewise resuspended but at 5×10^8 platelets/ml. The protocol used for lactoperoxidase-catalyzed ^{125}I-labeling was based on the method of Phillips and Poh Agin (22). To 1 ml of each platelet suspension were added sequentially 0.5 mCi ^{125}I, 10 μl 0.25 mM lactoperoxidase dissolved in the platelet suspension buffer, and finally at 10-s intervals, 5×12 μl 1 mM H_2O_2 also prepared in the platelet suspension buffer. The

labeled platelets were sedimented and washed twice before being resuspended for SDS-PAGE analysis.

SDS-PAGE procedures

Washed unlabeled or ^{125}I-labeled control platelets were resuspended at 2×10^9 platelets/ml in 0.01 M Tris-HCl, pH 7.0, containing 0.15 M NaCl, 3 mM EDTA and 5 mM *N*-ethylmaleimide. B-S platelets were similarly resuspended, but at a concentration of 5×10^8 platelets/ml. A one-fifth volume of 12% SDS was added and the samples heated at 100°C for 5 min. After cooling, aliquots were taken for protein analysis (23) and the rest was frozen at −20°C. Disulfide bond reduction was performed immediately before electrophoresis by incubating the samples at 37°C for 1 h in the presence of 5% vol/vol 2-mercaptoethanol.

GP analysis. SDS-PAGE was performed using 10.5 × 0.5-cm polyacrylamide rod gels and the Laemmli (24) buffer systems. Each sample contained 400 μg platelet protein and was mixed with 20 μl 50% vol/vol glycerol containing 0.0025% wt/vol bromophenol blue tracking dye before being applied to the gel. An internal standard for the PAS reaction, 5 μg orosomucoid dissolved in 2% SDS, was added to the sample before electrophoresis and before reduction. Reduced samples were analyzed on 6% acrylamide (0.2% bis) separating gels, nonreduced samples on 7% acrylamide (0.2% bis) separating gels. Each gel was overlayered with a 1.0 cm 3.5% acrylamide stacking gel. Electrophoresis was performed overnight at 25 V and continued until the tracking dye approached within 0.5 cm of the base of the tube. After electrophoresis the gels were washed overnight in 25% vol/vol isopropanol: 10% vol/vol acetic acid. GP were located by the PAS reaction (25); the Schiff reagent was prepared fresh each month according to the procedure of Segrest and Jackson (26). The stained gels were stored at 4°C in 7% acetic acid and densitometrically scanned within 24 h using a Vernon Recording Photometer (Vernon Integrating Photometers, Paris, France) equipped with a green (Wratten No. 7) filter.

Polypeptide analysis. Samples were prepared and disulfide bonds reduced exactly as described for the GP analysis. SDS-PAGE was performed using 7.0–12.0% exponential gradient acrylamide slab gels of dimensions 10 (height) × 14 (length) × 0.15 cm (thickness). The ratio (g:g) of bis to acrylamide was maintained at 0.027 throughout the gradient. A 1-cm 3.0% acrylamide stacking gel was also used. The buffer systems of Laemmli (24) were again used and electrophoresis performed overnight at 25 V in a Bio-Rad model 220 vertical slab electrophoresis cell. Samples containing 100 μg protein were analyzed. Polypeptides were located using Coomassie Blue R 250 (27). Stained gels were incubated for 1 h in 10% vol/vol acetic acid containing 1% vol/vol glycerol and dried onto filter paper using a Bio-Rad model 224 Gel Slab Dryer. ^{125}I-labeled polypeptides were located by autoradiography, the dried gels being left in contact with Kodak X-Omat MA film at room temperature for up to 2 wk, after which the films were processed according to the manufacturer's instructions.

Two-dimensional SDS-PAGE analysis. This was performed using an adaptation of the nonreduced/reduced two dimensional system of SDS-PAGE analysis as applied to platelets by Phillips and Poh Agin (22). First dimension SDS-PAGE was performed using 7% acrylamide (0.2% bis) tube gels and nonreduced samples as outlined in the GP analysis section, 100 μg SDS-solubilized platelet protein now being applied to the gel. After electrophoresis the unstained gels were agitated for 1 h at room temperature in the presence of 0.01 M Tris, pH 7.0, containing 3 mM EDTA and 5% vol/vol 2-mercaptoethanol. The second dimension electrophoresis was performed using 7–12% exponential gradient acrylamide slab gels as outlined in the section polypeptide analysis. One exception was the substitution of the outer standard glass plate in the gel forming apparatus with a plate with a bevelled upper edge (Bio-Rad Laboratories). The gel containing the now-reduced polypeptides was layered onto the second dimension gel, over the 3% stacking gel. The rod gel was fixed in position by the addition of a small volume of 1% agarose dissolved by warming in 10 mM Tris, pH 7.0. A small plastic template was attached at the right-hand side of the layered polyacrylamide tube gel before addition of the agarose. An aliquot (100 μg protein) of reduced proteins of the sample being analyzed was added to the cavity in the agarose that formed when the template was removed. After electrophoresis the gels were stained with Coomassie Blue R 250. On occasion, PAS-staining was first performed to locate the position of the major GP. The detection of ^{125}I-labeled proteins by autoradiography was performed as described in the section polypeptide analysis.

Molecular weight determinations. Apparent molecular weights of the platelet GP and polypeptides were determined by comparing their relative migration during SDS-PAGE with that of the following standard proteins: myosin heavy chain (200,000 mol wt), β-galactosidase (116,500 mol wt), phosphorylase b (94,000 mol wt), bovine serum albumin (68,000 mol wt), ovalbumin (43,000 mol wt), carbonic anhydrase (30,000 mol wt), soybean trypsin inhibitor (21,000 mol wt) and lysozyme (14,300 mol wt). Mixtures containing 2.5 μg of each protein were incubated with SDS and 2-mercaptoethanol and SDS-PAGE performed exactly as described for the platelet samples. Measurements were made to the leading edge of the stained bands.

Neuraminidase digestion

Washed normal human platelet suspensions were resuspended at 1×10^9 platelets/ml in 0.01 M sodium phosphate, pH 7.4, containing 0.15 M NaCl. Preincubation at 37°C for 2 min was followed by the addition of 0.05 vol neuraminidase (500 U/ml) in 0.05 M sodium acetate buffer, pH 5.5, containing 0.154 M NaCl and 0.009 M $CaCl_2$. The incubation was continued with agitation for 15 min, at which time the digestion was stopped by the addition of 4 mM EDTA. The platelets were sedimented and washed once in 0.01 M Tris-HCl, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA and then resuspended for lactoperoxidase-catalyzed (^{125}I) iodination. Total platelet sialic acid was estimated by the thiobarbituric acid method of Aminoff (28) following its release by hydrolysis with 0.1 N H_2SO_4 at 80°C for 1 h. Neuraminidase-released sialic acid was measured without prior acid hydrolysis. Optical densities were read at both 532 and 549 nm and adjusted for the presence of 532-nm-absorbing impurities (29).

RESULTS

B-S platelets. As is characteristic for the B-S syndrome each of the patients possessed a low circulating platelet count at the time of study (Table I). The protein content of the isolated platelets was increased, ranging from two to four times the normal amounts. Approximately 30% less sialic acid/mg protein was located in the platelets of the two patients where it was estimated.

Platelet GP nomenclature. The four major PAS-staining membrane GP are termed Ib, IIb, IIIa, and IIIb according to the criteria described by Nurden and Caen (30) and as previously used by us. GP Ig refers

TABLE I
Laboratory Studies on B-S Platelets

Patient	Whole blood platelet count	Platelet protein	Platelet sialic acid
	10^3/cu/mm	*mg/10^9 platelets*	*μg/mg protein*
N.V. (3)	88	16.5	5.0
D.S. (1)	90	17.2	ND
W.B. (1)	125	7.9	ND
R.B. (2)	25	8.4	4.8
Controls (20)	160–400*	3.85±1.22§	7.0±0.8§

The numbers in parentheses represent the number of times each patient has been examined. The values given for each patient are the mean of the results obtained, ND (not determined).
* Range.
§ Mean±SD.

to the intracellular, granule localized GP that has been termed "thrombin sensitive protein" (31) or "thrombospondin" (32). Other membrane GP are given the nomenclature established by Phillips and Poh Agin (22) where it was clearly apparent that their electrophoretic mobilities, with and without disulfide bond reduction, were comparable.

Platelet GP analysis. Preliminary studies established that the optimal separation of the major PAS-staining platelet GP was achieved using the Laemmli (24) system of electrophoresis. Rod gels were used to facilitate densitometric scanning. The optimal acrylamide concentration for the analysis of reduced samples was found to be 6% acrylamide (0.2% bis). Fig. 1 compares the GP profiles of reduced samples of normal human platelets and those of the B-S patient (R.B.). The B-S platelet profile shows a specific and severe reduction in the PAS-staining intensity of the Ib band. Note that the absence of this band allows the full appearance of a PAS-peak, band 4, present as a slowly migrating shoulder to GP Ib in the normal platelet profile. Band 4 has an apparent molecular weight of 160,000 and for reasons referred to later probably represents the PAS stain of GP Ia and IIa.

As detailed by Phillips and Poh Agin (22), the platelet membrane GP contain differing amounts of inter- or intramolecular disulfide bonds, thus their rate of migration during electrophoresis depends on the state of reduction of these bonds. Fig. 2 compares the GP profiles of nonreduced normal human and B-S platelets. A clearly apparent absence of staining in the Ib position was again a feature of the B-S platelet profile. Similar GP profiles were observed for the platelets of all four patients studied. Inasmuch as the added orosomucoid internal standard tended to migrate nearer to the tracking dye front on electrophoresis of nonreduced samples, an increased 7% acrylamide (0.2% bis) concentration was used for their analysis. When nonreduced samples were analyzed, it was necessary to block intrinsic membrane sulfhydryl groups with *N*-

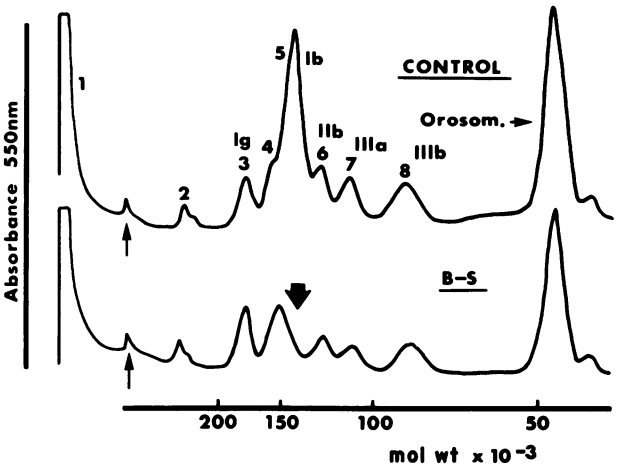

FIGURE 1 GP profile of B-S platelets after disulfide bond reduction. Washed normal human platelets and those isolated from B-S patient (R.B.) were solubilized in 2% SDS as described in the Methods. Constituent disulfide bonds were reduced by incubating the samples for 1 h at 37°C in the presence of 5% vol/vol 2-mercaptoethanol. Electrophoresis was performed using 6% acrylamide rod gels and the procedure of Laemmli (24), 400 μg platelet protein was applied to each gel together with 5 μg orosomucoid (Orosom). The GP were located by the PAS reaction, the orosomucoid acting as an internal standard. Typical densitometric profiles are illustrated. The separating gel origin is marked (↑), the profile to the left of this mark shows the PAS stain in the stacking gel.

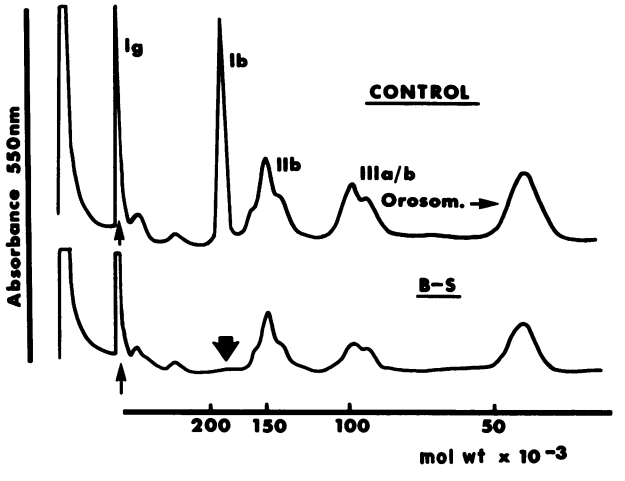

FIGURE 2 GP profile of B-S platelets in the absence of disulfide bond reduction. Washed normal human platelets and those isolated from B-S patient (W.B.) were dissolved by heating at 100°C for 5 min in the presence of 2% SDS and 5 mM *N*-ethylmaleimide. SDS-PAGE was performed using 7% acrylamide rod gels as described in the legend to Fig. 1. Typical densitometric profiles of PAS-stained gels are illustrated.

ethylmaleimide before heating samples at 100°C with SDS to prevent formation of high molecular weight aggregates of both membrane proteins and GP that fail to penetrate the separating gel. A similar observation has previously been made (22).

Platelet protein composition. Analysis of the B-S platelet proteins was performed on 7–12% gradient acrylamide slab gels allowing the detection of the major polypeptides within a molecular weight range of 400,000 to 20,000. The polypeptide profiles of the platelets of two patients are illustrated in Fig. 3. No gross protein abnormalities were observed. In particular, three large polypeptides with apparent molecular weights of 250,000, 230,000, and 200,000 were normally detected. These polypeptides, which have been shown to be highly sensitive to degradation by an endogenous platelet calcium-sensitive protease (33), have been reported to be modified in B-S platelets (14). As shown in Table I, the total protein content of B-S platelets was increased relative to normal human platelets. Fig. 3 shows that this increase, whether it was two- (W.B.) or fourfold (N.V.) was the result of a general augmentation of the platelet proteins. We saw no sign of plasma protein absorption. The position of GP Ib was located by prior PAS-staining and is shown in Fig. 3. A faint Coomassie Blue band normally observed in this position was missing from the B-S platelet polypeptide pattern. In contrast, the Coomassie Blue band given by GP IIb, which is only separated from other comigrating polypeptides on nonreduced gels (22), was present and possessed a normal migration.

Analysis of ^{125}I-labeled platelets. The surface proteins of platelets isolated from patients (N.V., R.B., and W.B.) have been labeled with ^{125}I by the lactoperoxidase-catalyzed procedure and the labeled proteins analysed by SDS-PAGE. A comparison of different procedures showed that the best resolution was achieved by autoradiography following the analysis of platelet samples on 7–12% gradient acrylamide slab gels. A typical analysis as performed on the platelets of patient W.B. is illustrated in Fig. 4. Identification of the labeled bands was aided (*a*) by correlating the peaks of radioactivity with the individual PAS-stained GP as located on slab or rod gels, (*b*) by the analysis of ^{125}I-labeled platelets of a patient with Glanzmann's thrombasthenia, whose platelets were specifically deficient in IIb and IIIa (31), and (*c*) by direct comparison with the profiles given by Phillips and Poh Agin (22). Analysis of reduced B-S platelet samples showed zones of radioactivity apparently normally associated with IIb and IIIa, the surface GP maximally labeled with ^{125}I by the lactoperoxidase-catalyzed procedure. However, the close migration of Ib, Ia, and IIa prevented a precise evaluation of their ^{125}I-labeling intensities. In contrast, analysis of nonreduced samples, which more clearly separated Ib from the other major membrane GP (compare also Figs. 1 and 2), showed a specific abnormality in the Ib position.

Two-dimensional SDS-PAGE analysis. The nonre-

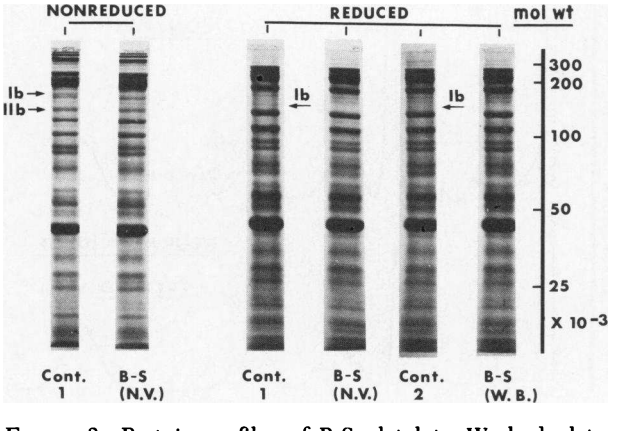

FIGURE 3 Protein profiles of B-S platelets. Washed platelets from two control donors and from B-S patients (N.V. and W.B.) were solubilized with SDS and aliquots (100 μg protein) subjected to SDS-PAGE after (reduced samples) or in the absence of disulfide bond reduction (nonreduced samples). SDS-PAGE was performed using 7–12% gradient acrylamide slab gels and the procedure of Laemmli (24). Proteins were detected with Coomassie Blue R 250. The position of GP Ib on the control patterns was detected by PAS-staining and is shown. The position of the protein band corresponding to IIb is likewise marked on the nonreduced profiles.

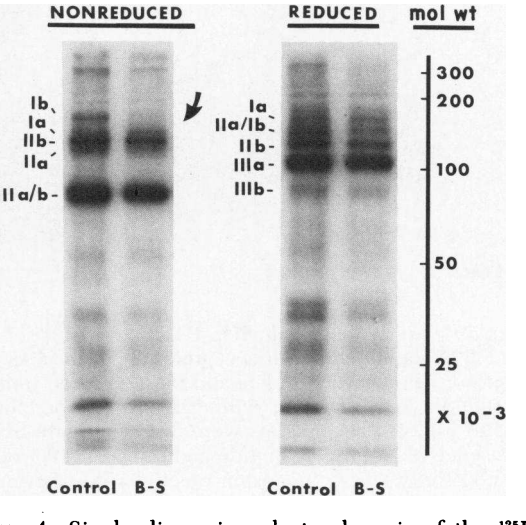

FIGURE 4 Single dimension electrophoresis of the ^{125}I-labeled surface proteins of B-S platelets. Washed suspensions of normal human platelets and those isolated from the B-S patient (W.B.) were incubated in the presence of ^{125}I and the surface proteins labeled by the lactoperoxidase-catalyzed iodination procedure as described in the Methods. Aliquots of both reduced and nonreduced SDS-solubilized protein (100 μg) were analysed by SDS-PAGE on 7–12% exponential gradient acrylamide slab gels as described in the legend to Fig. 3. ^{125}I-labeled proteins were detected on protein-stained, dried gels by autoradiography.

duced/reduced two-dimensional system of SDS/PAGE of Phillips and Poh Agin (22) uses the different contents of inter- or intramolecular disulfide bonds within the individual membrane GP to improve the resolution achieved using single dimension electrophoresis alone. Fig. 5 illustrates the analysis of ^{125}I-labeled normal and B-S platelets by this procedure. Autoradiographs of dried, Coomassie Blue-stained gels are shown. An absence of ^{125}I-labeling in the Ib position is the striking feature of the autoradiograph of the B-S platelet gel and confirms the finding illustrated for the platelets of the patient (W.B.) in Fig. 4. Furthermore, zones of radioactivity corresponding to GP Ia and IIa were clearly detected. Two low molecular weight spots given by the β-subunits of Ic and IIb (22) were also normally present. Neither in our studies nor those reported by Phillips and Poh Agin (22) has the β-subunit of GP Ib been shown to be labeled with ^{125}I during lactoperoxidase-catalyzed iodination of normal human platelets. It was, however, clearly detected by Coomassie Blue staining. No spot corresponding to the β-subunit of Ib was located when B-S platelets were so analyzed (data not shown).

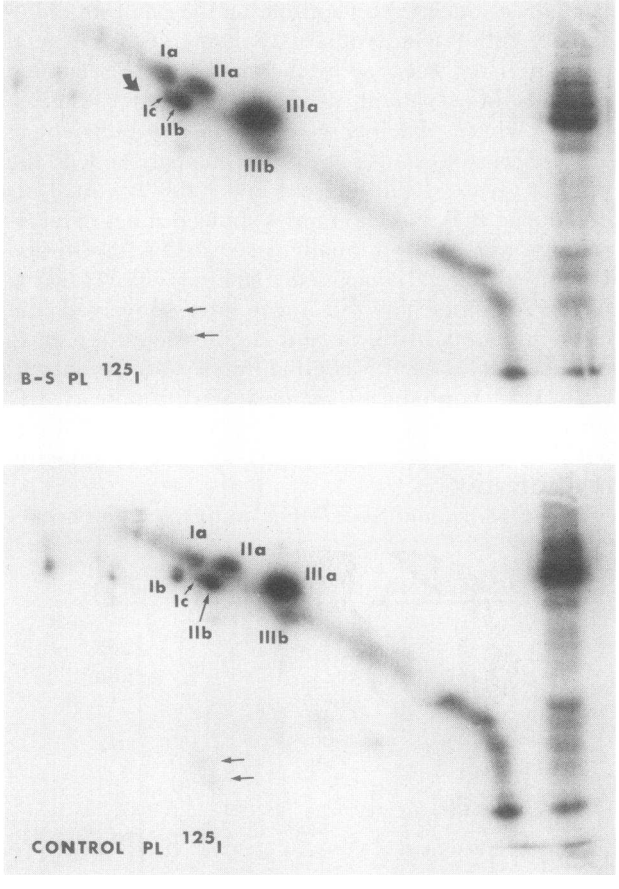

FIGURE 5 Two-dimensional SDS-PAGE analysis of ^{125}I-labeled B-S platelet (PL) surface proteins. Washed suspensions of normal human platelets and those isolated from the patient (N.V.) were labeled with ^{125}I as described in the legend to Fig. 4. The platelets were dissolved with SDS in the presence of *N*-ethylmaleimide and samples (100 μg protein) subjected to first dimension electrophoresis using 7% acrylamide rod gels as described in the legend to Fig. 2. After electrophoresis, the unstained gels were incubated in the presence of 5% vol/vol 2-mercaptoethanol and a second dimension electrophoresis of the now reduced proteins performed on 7–12% gradient acrylamide slab gels. A single sample of reduced ^{125}I-labeled proteins (100 μg) of the sample being analysed was also applied to the right hand side of the slab gel. Protein-stained gels were dried onto filter paper and the ^{125}I-labeled proteins detected by autoradiography. Two weakly labeled low molecular weight polypeptides are marked (←) in the lower part of the gel, these correspond to the β-subunits of Ic and IIb respectively (22).

Analysis of neuraminidase-treated platelets. Normal human platelet suspensions were incubated with neuraminidase under conditions which gave rise to the maximal release of surface sialic acid residues. The surface proteins of the desialated platelets, which had lost ~50% of their total platelet sialic acid content, were then labeled with ^{125}I by the lactoperoxidase-catalyzed procedure. Fig. 6 shows a PAS-profile typical of those obtained for neuraminidase-treated platelets following SDS-PAGE. A significant decrease in the PAS-staining capacity of Ib was observed, however, the GP was still a major contributor to the PAS-profile. Only minor modifications in the PAS-staining capacity

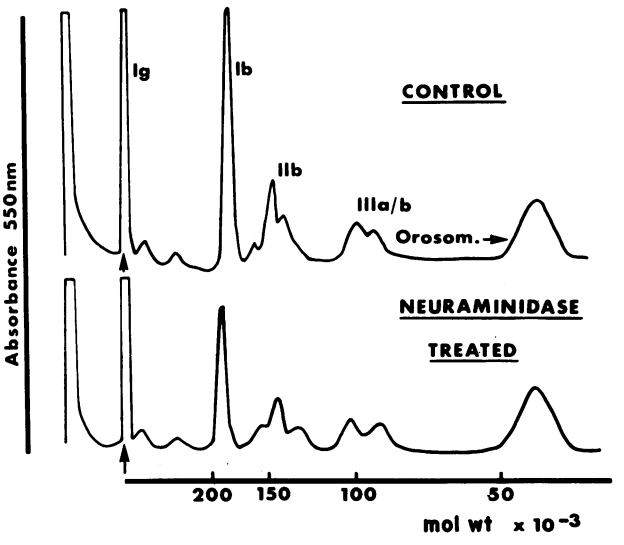

FIGURE 6 GP profile of neuraminidase-treated normal human platelets. Washed platelet suspensions were incubated for 15 min at 37°C in the presence or absence of neuraminidase (25 U neuraminidase/10^9 platelets). Samples of the control and neuraminidase-treated platelets were dissolved by SDS and analyzed without reduction on 7% acrylamide rod gels as described in the legend to Fig. 2. GP were detected by the PAS-reaction, typical densitometric profiles are shown.

of the other membrane GP were detected. The residual Ib exhibited a slightly slower rate of migration during electrophoresis whereas that of IIb was increased. The altered migration of Ib suggested that the observed peak represented desialated, or partially desialated, Ib rather than a GP fraction inaccessible to the action of the neuraminidase.

Analysis of the ^{125}I-labeled proteins by autoradiography clearly showed an ^{125}I-labeled band in the GP Ib region of the gel although, as expected from the PAS-staining, the band had a slightly slower migration (Fig. 7). The identity of this band as Ib was confirmed using the nonreduced/reduced two dimensional SDS-PAGE system (data not shown). Apart from mobility changes, remarkably little alteration was observed in the distribution of ^{125}I among the surface proteins of the desialated normal platelets. The protein profiles of the neuraminidase-treated platelets also showed no modifications, except for the altered rates of migration of Ib and IIb now more clearly apparent owing to the greater sharpness of the protein-stained bands.

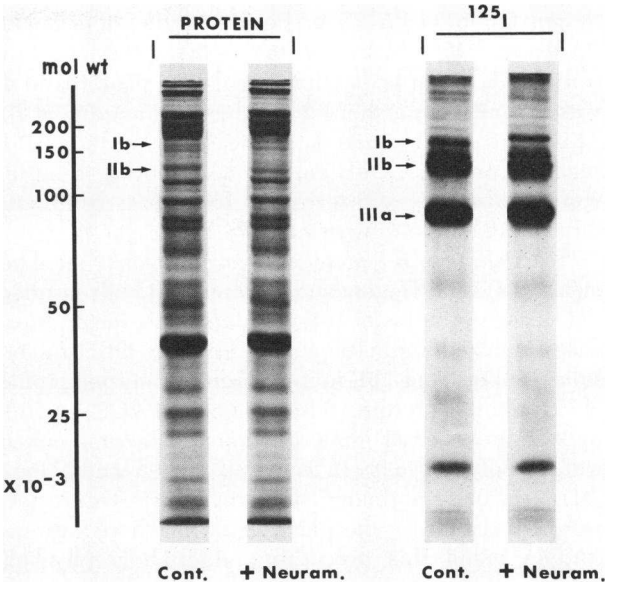

FIGURE 7 Protein and ^{125}I-labeling profiles of neuraminidase-treated normal platelets after single dimension electrophoresis. Washed platelet suspensions were incubated in the presence or absence of neuraminidase as described in the legend to Fig. 6. The control (Cont.) and neuraminidase (Neuram.) treated platelets were then incubated in the presence of ^{125}I and the surface proteins labeled using the lactoperoxidase-catalyzed procedure. Aliquots (100 μg protein) of nonreduced SDS-solubilized platelets were analyzed by SDS-PAGE on 7–12% gradient acrylamide slab gels. The gels were stained for protein, dried and analyzed by autoradiography.

DISCUSSION

Despite the fact that a number of investigators have reported studies on the GP and polypeptide composition of B-S platelets (3, 11, 13–16, 21, 30, 34–36), the conflicting data reported in the literature has made it difficult to precisely interpret the nature of the molecular lesion(s) of these functionally defective platelets. Our current work has been designed to clarify the nature of the structural abnormalities of B-S platelets.

Characterization of normal human platelet GP. In view of the difficulty in isolating well characterized membrane fractions from the platelets of patients with moderate to severe thrombocytopenia (each of the patients studied had a low circulating platelet count, Table I) our investigation has been restricted to the analysis of unfractionated washed platelet suspensions. Preliminary studies compared the GP resolution obtained using SDS-PAGE procedures incorporating a continuous SDS-phosphate buffer system similar to that used for platelet GP analysis by George et al. (37), with procedures incorporating the discontinuous SDS-Tris glycine buffer system as employed by Phillips and Poh Agin (22). Emphasis was placed on obtaining PAS profiles that allowed the quantitative evaluation of the major PAS-bands by densitometric scanning. The best resolution of the major PAS-staining GP was achieved using the discontinuous buffer system, reduced samples, and 6% acrylamide separating gels. In comparison with the GP profiles of reduced, whole platelets given by George et al. (37), we have obtained an improved separation of GP Ig from Ib and of GP IIIa from IIIb.

B-S platelet GP. The predominant feature of the GP profile of the platelets of each of the four patients studied was the total absence of PAS-staining in the position normally occupied by GP Ib. The characterization of this defect was aided by the analysis of both reduced and nonreduced samples. As defined by Phillips and Poh Agin (22) Ib is a high molecular weight GP composed of two subunits. Incubation of the parent molecule with 2-mercaptoethanol results in the cleavage of intermolecular disulfide bonds and the separation of a small (22,000 mol wt) β subunit from a large (148,000 mol wt) α subunit. In terms of this nomenclature the PAS band termed Ib in the GP profile of reduced, normal platelets in Fig. 1 is the α subunit of Ib. The labeled Ib in the GP profile of nonreduced, normal platelets in Fig. 2 is the parent molecule.

Jamieson et al. (35) isolated membranes from the platelets of two B-S patients and reported a specific defect but that 40% of the PAS-stain was located in the GP I position following SDS-PAGE. Comparisons between our results and those of Jamieson et al. (35) may suggest heterogeneity in the extent of the Ib lesion observed between different patients. A possible contribution of Ia and IIa to the PAS-stain located in the GP I position was not, however, taken into account by Jamieson et al. (35) who analyzed reduced samples by a single

dimension SDS-PAGE procedure giving four major PAS-stained bands. In our studies using reduced control platelet samples an additional PAS-peak (band 4) was separated as a shoulder to the trailing edge of Ib. This band was a marked feature of the reduced B-S platelet profiles. Only using nonreduced samples were we able to clearly resolve Ib from other membrane GP during single dimension SDS-PAGE.

^{125}I-Labeling of B-S platelet surface proteins. Jenkins et al. (14) and Hagen and Solum (34) both studied the surface proteins of B-S platelets using lactoperoxidase-catalyzed iodination and reported differing results. Jenkins et al. (14) found a highly abnormal profile of ^{125}I-labeled proteins following SDS-PAGE, including the presence of numerous labeled low molecular weight polypeptides. In contrast, Hagen and Solum (34) described a normal ^{125}I-labeling profile. Our results were consistent for the platelets of the three patients studied using this procedure; autoradiography following single and two-dimensional SDS-PAGE showed an absence of labeling in the Ib position. The other major ^{125}I-labeled surface GP as detailed by Phillips and Poh Agin (22) were all normally detected. We located none of the changes previously described for B-S platelets by Jenkins et al. (14).

In their studies Hagen and Solum (34) detected ^{125}I-labeled polypeptides by slicing PAS-stained rod gels and measured the radioactivity in the slices using a gamma counter. We followed the same procedure initially but noted that accurate evaluation of Ib was difficult owing to the small percentage of the total radioactivity located in the GP I region. Subsequently, we performed autoradiography after the separation of the labeled proteins on gradient slab gels. Exposure times of up to 2 wk amplified the detection of the less-strongly labeled surface proteins. Furthermore, in view of the increased protein content of the B-S platelets (Table I) and their increased size (1, 6), at least under the conditions of this study (38), we decreased the concentration of B-S platelets during the lactoperoxidase-catalyzed iodination (see Methods). With these procedures, a clear absence of radioactivity in the Ib position was observed either after the analysis of ^{125}I-labeled B-S platelets by the two-dimensional SDS-PAGE system or after the analysis of nonreduced samples on single dimension gradient acrylamide gels.

Relationship between Ib and glycocalicin. Solum and his co-workers (17, 39) have provided immunochemical evidence that Ib is the membrane GP precursor of glycocalicin, a large (148,000 mol wt) glycopolypeptide composed of ~60% by weight carbohydrate, that is solubilized from the platelet surface during platelet homogenization (40). Our present results indirectly support this finding in that Ib was the only major PAS-staining GP to show a severe reduction in staining on analysis of B-S platelets, which have been shown in several studies to lack glycocalicin (15, 16, 21, 35). George et al. (37) have expressed a similar view following the analysis of normal platelets before and after glycocalicin loss. In contrast, Nachman et al. (41) have claimed that Ib and glycocalicin give different peptide maps following trypsin digestion and are different GP although absolute proof for the identification of Ib was not given.

Examination of neuraminidase-treated control platelets. In confirmation of the original report of Gröttum and Solum (12) the platelets of the two B-S patients examined showed a decreased sialic acid content. However, despite the removal of ~50% of the total sialic acid content of normal human platelets by neuraminidase treatment before ^{125}I-labeling little change was induced in the pattern of the ^{125}I-labeled GP as observed after SDS-PAGE. A clear band or spot in the Ib position remained on the autoradiographs. McGregor et al. (36) have suggested that there is either a severe reduction or an altered glycosylation of GP Ia and Ib in B-S platelets. Our results point to the presence of Ia on the B-S platelet surface as this GP was apparently normally labeled with ^{125}I during lactoperoxidase-catalyzed iodination. In contrast, any structural defects in Ib must be severe as we were unable to locate it by PAS staining, ^{125}I-labeling, or, in previous studies, by crossed immunoelectrophoresis (15). The presence of a molecular deficiency of Ib in B-S platelets must be considered. Further studies are now being performed to examine the state of glycosylation of GP Ia in the platelets of our patients, meanwhile its presence in their platelets has been recently confirmed by crossed immunoelectrophoresis.[2]

In agreement with McGregor et al. (42) we observed that GP Ib of normal human platelets migrated with an increased apparent molecular weight after neuraminidase treatment (see Figs. 6 and 7). This slower rate of migration is probably due to the normal contribution of the anionic charge of sialic acid to the migration of Ib during SDS-PAGE.

Possible origin of the B-S platelet defect. Our results emphasize that B-S platelets contain a specific and characteristic membrane lesion. We have obtained no evidence for the large scale changes in polypeptide composition as attributed to B-S platelets by Jenkins et al. (14). Furthermore, we failed to detect signs of the presence of widespread endogenous protease activity in B-S platelets as proposed by Shulman and Karpatkin (16). The possible presence of exogenous protease activity either in the bone marrow or plasma was also considered. If this was present then presumably the

[2] Kunicki, T. J., A. T. Nurden, D. Pidard, N. R. Russell, and J. P. Caen. Characterisation of platelet antigens giving rise to individual immunoprecipitates in CIE. Manuscript submitted for publication.

protease would be free to interact with the surface of other blood cells. Analysis of membranes isolated from erythrocytes of each patient, however, revealed no signs of GP degradation (unpublished results). If protease activity is the cause of the B-S platelet surface lesion then the protease must have a narrow specificity and a particular affinity for platelet GP Ib.

The alternative hypothesis, therefore, is that a direct genetic defect at a stage in the synthesis of Ib may be the cause of the B-S platelet membrane lesion. Studies on the megakaryocytes of B-S patients may be required to locate the origin of the abnormality. Meanwhile we emphasize that the B-S syndrome is a distinct congenital platelet disorder characterized by a distinctive membrane GP abnormality.

ACKNOWLEDGMENTS

These studies were supported by grant CRL 78.5.128.1 from Institut National de la Santé et de la Recherche Medical (INSERM), France, and by a grant from the Thyssen Foundation, West Germany.

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