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Inherited Abnormalities of Platelet Glycoproteins [and Discussion]

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Inherited abnormalities of platelet glycoproteins

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Glanzmann's thrombasthenia and the Bernard–Soulier syndrome are inherited blood disorders characterized by abnormalities in different aspects of platelet function during haemostasis. Platelets from patients with thrombasthenia do not aggregate in response to the normal physiological platelet aggregation inducing stimuli, while Bernard–Soulier platelets have a reduced capacity to adhere to exposed subendothelium. Deficiencies of different membrane glycoproteins have been located in the platelets of both disorders and suggest specific roles for membrane glycoproteins in different aspects of platelet function.

Introduction

When platelets aggregate, a stimulus received at a receptor within the platelet plasma membrane sets in motion a chain of events that results in the formation of bonds between adjacent cells. Similarly, when platelets adhere to subendothelial components exposed during vessel injury, the process is initiated by the interaction of specific stimuli with externally orientated membrane receptors and concluded with the formation of cohesive forces exposed at the platelet surface. Cell contact interactions in mammalian cell systems are often mediated by membrane glycoproteins (Nicolson 1976), and glycoproteins have been shown to be major components of the normal human platelet surface (Phillips & Poh Agin 1977*a*). Evidence supporting a possible role for membrane glycoproteins in platelet surface contact interactions has come from studies performed on platelets isolated from patients with inherited disorders of platelet function.

Membrane glycoprotein composition of normal human platelets

Phillips (1972) and Nachman & Ferris (1972) initially described three glycoproteins after SDS–polyacrylamide gel electrophoresis of human platelet membranes solubilized by the detergent sodium dodecyl sulphate (SDS). These glycoproteins, in the molecular mass range M_r = 150000–90000, were located by staining for carbohydrate by using the periodate–Schiff reaction. Phillips (1972) termed the glycoproteins I, II and III and established the convention whereby the glycoprotein (GP) with the highest molecular mass was termed GPI, the next GPII and so on. Subsequently, it was shown by using lactoperoxidase-catalysed ^{125}I-labelled platelets that GPII contained two components that were denoted GPIIa and IIb (Phillips *et al.* 1975).

Thus the further convention was established that as heterogeneity within the major periodate–Schiff-staining bands became apparent, the newly resolved species within each molecular mass class were each given a different letter suffix. The glycoprotein composition of the platelet plasma membrane is now known to be complex (Phillips & Poh Agin 1977*a*; Clemetson *et al.*

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1979), with the number of components observed depending largely on the sensitivity of the analytical approach used.

Some characteristics of the platelet membrane glycoproteins are illustrated in figure 1. Two different surface-labelling techniques have been used to incorporate a radioactive label into those molecules with part of their structure exposed at the human platelet surface. Lactoperoxidase-catalysed iodination has been used as a method for incorporating ^{125}I into the tyrosine residues of polypeptide chains exposed at the platelet surface. Alternatively, washed

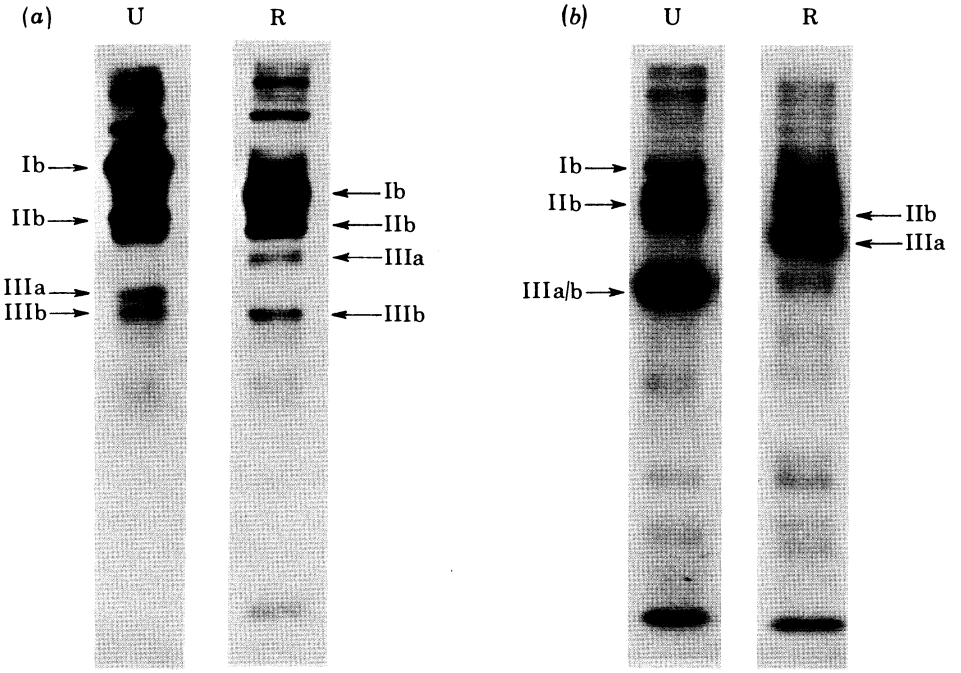

FIGURE 1. Identification of the surface-orientated membrane glycoproteins of normal human platelets after analysis by SDS–polyacrylamide gel electrophoresis of platelets whose surface proteins had been radiolabelled by two different surface labelling procedures. Washed human platelets were incubated (*a*) sequentially with neuraminidase, galactose oxidase and sodium [^{3}H]borohydride as described by McGregor *et al.* (1979), or (*b*) with lactoperoxidase and ^{125}I as described by Phillips & Poh Agin (1977*a*). The radiolabelled platelets were analysed by SDS–polyacrylamide gel electrophoresis on 7–12% gradient acrylamide slab gels in the absence of (unreduced; U) or after (reduced; R) disulphide bond reduction as detailed by Nurden *et al.* (1981). Radiolabelled proteins were located by fluorography (^{3}H) or by radioautography (^{125}I).

platelets have been treated sequentially with neuraminidase, galactose oxidase and sodium [^{3}H]borohydride with the result that ^{3}H has been incorporated into galactose and *N*-acetylgalactosamine residues of membrane glycoproteins. It may be noted that GPIb is labelled profusely with ^{3}H, probably as a result of its having a high carbohydrate content. In contrast, GPIIIa is weakly labelled with ^{3}H but strongly labelled with ^{125}I, suggesting that it has a lower sugar content and a large proportion of its protein structure exposed at the surface.

Also illustrated in figure 1 is the altered mobility of GPIb, IIb and IIIa following disulphide bond reduction. GPIb and IIb appear to be dimeric molecules, the subunits of which are linked by one or more disulphide bonds. Reduction results in the separation of a large, glycosylated polypeptide (α-chain) from a smaller polypeptide (β-chain) which may or may not be glycosylated (Phillips 1979). In contrast, GPIIIa appears to contain intramolecular disulphide bonds. Although the platelet plasma membrane contains many glycosylated components other

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than those labelled in figure 1, the four glycoproteins highlighted on this figure are those that have been best characterized to date; they will form the basis of this review. The surface orientation of the platelet membrane glycoproteins is confirmed by their rapid degradation when proteases are added to washed platelet suspensions (Nurden & Caen 1975; Kunicki & Aster 1979). Furthermore, the presence of a layer rich in bound carbohydrate at the outer surface of the platelet plasma membrane has been demonstrated by cytochemical procedures used in combination with electron microscopy (Behnke 1968).

Glanzmann's thrombasthenia

A disease with an autosomal recessive inheritance, Glanzmann's thrombasthenia (G.t.) is characterized by the absence of platelet aggregation in response to ADP and all physiological aggregation-inducing agents (for a review see Hardisty 1977). As discussed elsewhere (Nurden & Caen 1979), the basic defect appears to lie in the inability of G.t. platelets to form the platelet–platelet cohesive forces that conclude the aggregation mechanism. The platelets of most patients are also unable to support clot retraction (Caen *et al.* 1966). Occasionally a modified clot retraction is observed, although the platelet aggregation defect remains unchanged. These patients have been described as type II thrombasthenia by Caen (1972).

Membrane fractions isolated from thrombasthenic platelets were first studied by Nurden & Caen (1974), who noted the presence of glycoprotein abnormalities. SDS–polyacrylamide gel electrophoresis followed by periodate–Schiff staining revealed marked reductions in the staining intensity normally observed in the GPII and III regions of the gel. Molecular differences in the surface composition of thrombasthenic platelets were confirmed by Phillips *et al.* (1975) with the use of the lactoperoxidase-catalysed procedure for labelling surface proteins with ^{125}I or ^{131}I.

Platelets from a larger number of thrombasthenic patients have now been examined by a number of different radiolabelling and SDS–polyacrylamide gel electrophoresis procedures (Phillips & Poh Agin 1977*b*; White *et al.* 1978; Hagen & Solum 1978; Kunicki & Aster 1978; Nurden & Caen 1979). Accumulated evidence from these studies suggests severe molecular deficiencies of GPIIb and IIIa as a specific membrane defect of thrombasthenic platelets. This finding is illustrated in figure 2.

Kunicki & Aster (1978) first showed that the platelet-specific alloantigen Pl^{A1} (Zw^a) was deleted from platelets in thrombasthenia; they then proved (Kunicki & Aster 1979) that the Pl^{A1} antigenic marker was associated with GPIIIa on normal human platelets. Further support for this conclusion was provided by Van Leeuwen *et al.* (1979), who showed that the allelic counterpart of Pl^{A1}, Pl^{A2} (Zw^b), was also missing from G.t. platelets.

Triton X-100 solubilized normal human and thrombasthenic platelet proteins were studied by crossed immunoelectrophoresis (c.i.e.) by using a polyspecific rabbit antiserum against normal human platelets (Hagen *et al.* 1980). The treatment with the non-ionic detergent resulted in the solubilization of 90% of the total platelet protein. Two immunoprecipitates were observed to be missing from the patterns obtained by using G.t. platelets. In terms of the nomenclature used, these were denoted bands 16 and 24. Band 24 was identified as being given by platelet fibrinogen. Band 16 was shown to be heavily labelled with ^{125}I when normal human platelets whose surface proteins had been labelled with ^{125}I were analysed. Elution of band 16 from unstained agarose gels was followed by SDS–polyacrylamide gel electrophoresis.

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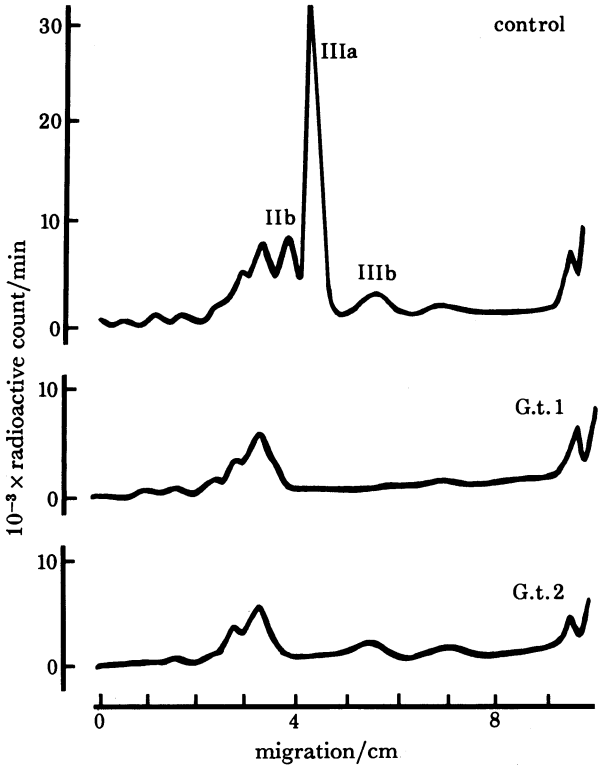

FIGURE 2. The abnormal surface structure of platelets isolated from patients with Glanzmann's thrombasthenia. The surface proteins of normal human platelets and those isolated from two patients with G.t. were labelled with ^{125}I by the lactoperoxidase-catalysed procedure. The radiolabelled platelets were analysed by SDS–polyacrylamide gel electrophoresis in 6% acrylamide tube gels after disulphide bond reduction as detailed by Nurden *et al.* (1981). After electrophoresis the gels were cut into 1 mm segments and the radioactivity contained in each segment measured in a γ-ray counter.

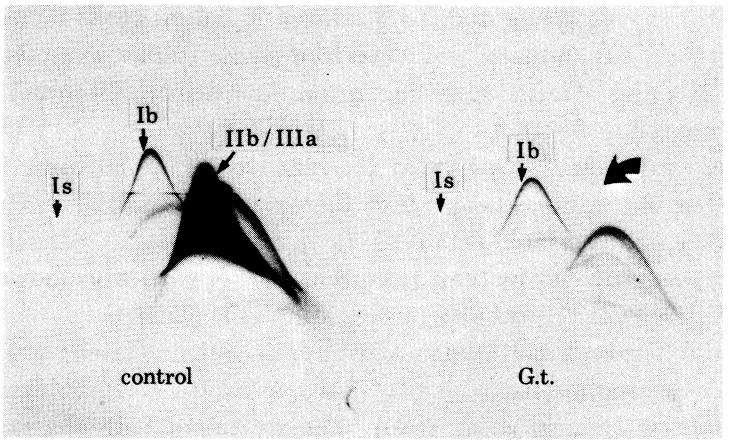

FIGURE 3. Analysis of the membrane glycoprotein composition of thrombasthenic platelets by crossed immuno-electrophoresis. The surface proteins of normal human platelets and those isolated from a patient with G.t. were labelled with ^{125}I by the lactoperoxidase-catalysed procedure. The platelets were solubilized in the presence of 1% Triton X-100 and c.i.e. performed according to the procedures detailed by Hagen *et al.* (1980). The immunoglobulin fraction of a multispecific rabbit antiserum prepared against washed, normal human platelets was incorporated into the agarose gel for the second dimension of electrophoresis. The immunoprecipitates containing radiolabelled antigens were located by radioautography.

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In this way GPIIb and IIIa were identified as the platelet antigens contained within the band 16 precipitate (Hagen *et al.* 1980). Their uniform distribution throughout the immunoprecipitation line suggested the presence of a membrane complex containing these antigens. Figure 3 illustrates the absence of band 16 from a radioautograph obtained after the analysis of radiolabelled G.t. platelets by c.i.e. Recent studies have shown the presence of intermediate levels of GPIIb/IIIa, $53 \pm 5\%$ (mean $\pm$ s.d.), in several kindred patients, with thrombasthenia (Kunicki *et al.* 1981*a*). The presence of intermediate levels of GPIIb/IIIa in the platelets of presumed heterozygotes for the thrombasthenia trait suggests a direct link between the inheritance of the disorder and the glycoprotein defect.

The above results all refer to thrombasthenic platelets that lack the ability to support either platelet aggregation or clot retraction. When platelets from a patient with type II thrombasthenia were analysed by c.i.e., band 24 (fibrinogen) was detected as normal, while band 16 (IIb/IIIa) was reduced to approximately 13% of the normal size (Hagen *et al.* 1980). Both functional and biochemical heterogeneity is exhibited in thrombasthenia.

Bernard–Soulier syndrome

The Bernard–Soulier (B.–S.) syndrome is a second platelet disorder with an autosomal recessive inheritance. This syndrome is characterized by a moderate to severe thrombocytopenia (low circulating platelet count), the presence of unusually large platelets on blood smears and a number of platelet function abnormalities (see Hardisty 1977). The primary haemostatic defect appears to be the inability of B.–S. platelets to adhere to the exposed subendothelial surface (Caen *et al.* 1976). Recent studies suggest that this is due to a defect in the interaction between the B.–S. platelet and microfibrils in the subendothelium (Legrand *et al.* 1980). As discussed elsewhere (Nurden & Caen 1979), the abnormality is probably related to the absence of agglutination of B.–S. platelets by ristocetin in the presence of normal human plasma, which in turn strongly suggests a defective interaction between the von Willebrand protein and its platelet receptor. Platelet aggregation rapidly follows platelet stimulation by ADP or collagen in the B.–S. syndrome; however, a decreased aggregation response to thrombin has been reported and characterized (Jamieson & Okumura 1978).

A specific glycoprotein abnormality in B.–S. platelets was first described by Nurden & Caen (1975), who observed a much decreased carbohydrate staining intensity of GPI after the analysis of isolated B.–S. platelet membranes by SDS–polyacrylamide gel electrophoresis. A greater resolution of the platelet surface components has been achieved in subsequent studies, which suggest a specific abnormality primarily affecting GPIb (Nurden & Caen 1979; Nurden *et al.* 1981). This finding is illustrated in figure 4. Here, SDS–solubilized normal human and B.–S. platelet glycoproteins have been analysed by SDS–polyacrylamide gel electrophoresis in the absence of disulphide bond reduction. As shown earlier (figure 1), GPIb is well separated from the other major carbohydrate-containing membrane glycoproteins when electrophoresis is performed without disulphide bond reduction.

The protein content of B.–S. platelets is increased twofold to fourfold (Nurden *et al.* 1981); however, analysis of their protein composition by using 7–12% acrylamide gradient gels showed normal polypeptide profiles. The increased protein content of B.–S. platelets is probably related to their increased granule content (Rendu *et al.* 1981) and to their increased size (see Hardisty 1977). When the surface composition of B.–S. platelets was studied by lactoperoxidase-

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catalysed iodination, ^{125}I was found to be incorporated into all of the normally labelled surface components with the exception of GPIb (Nurden *et al.* 1981). That B.–S. platelets were deficient in GPIb was further suggested by the studies of Hagen *et al.* (1980), who showed that a specific immunoprecipitate (band 13) was missing when B.–S. platelets were studied by c.i.e. Subsequent studies have clearly identified band 13 as being given by GPIb (T. Kunicki, unpublished observations).

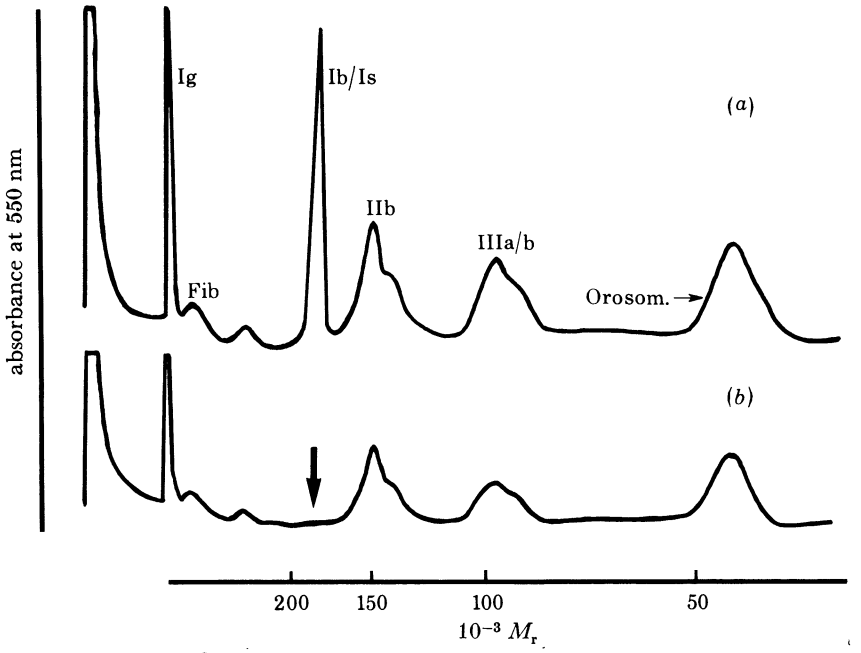

FIGURE 4. The abnormal membrane glycoprotein composition of Bernard–Soulier platelets. Washed normal human platelets (*a*) and those isolated from a patient with the B.–S. syndrome (*b*) were analysed by SDS–polyacrylamide gel electrophoresis on 7% acrylamide tube gels as detailed by Nurden *et al.* (1981) and in the absence of disulphide bond reduction. Glycoproteins were located by staining for carbohydrate with the periodate–Schiff reaction after electrophoresis. Densitometric profiles of the stained gels are illustrated.

The position of the immunoprecipitate given by GPIb is clearly shown on figure 3. Glycocalicin or GPIs are the names given to a glycopeptide of high molecular mass, derived from GPIb through the action of a protease that is active in platelet homogenates in the presence of divalent cations (Solum *et al.* 1980). GPIs is often clearly observed after c.i.e. as a fast migrating component giving an immunoprecipitate with a line of identity with GPIb. This precipitate may also be observed on figure 3. An antiserum prepared against purified glycocalicin has been used in previously reported c.i.e. studies (Hagen *et al.* 1980), where it was concluded that both GPIs and its precursor membrane glycoprotein were absent from the platelets of B.–S. patients. These, and our previous results (Nurden & Caen 1979), strongly suggest that the precursor of glycocalicin is GPIb.

In an earlier study, Kunicki *et al.* (1978) showed that B.–S. platelets, while possessing the Pl^{A1} antigen, lacked the receptor for quinine- or quinidine-dependent antibodies that was present on the platelets of all normal subjects tested. This receptor was subsequently shown to be associated with membrane GPIb or a structural analogue of this glycoprotein as isolated by chromatography on wheatgerm lectin affinity columns (Kunicki *et al.* 1981*b*). The absence of this receptor appears to be a specific characteristic of the B.–S. platelet surface.

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Current thoughts on the possible roles of membrane glycoproteins in normal human platelet function

The crucial question to be answered is whether the observed functional defects of thrombasthenic and Bernard–Soulier platelets are a direct result of glycoprotein deficiencies. Fibrinogen has long been known as an essential cofactor for platelet aggregation. Recent studies have shown that specific receptor sites for fibrinogen are exposed on the platelet surface after stimulation with ADP, adrenalin (Plow & Marguerie 1980) and probably other aggregation inducers. No such receptor sites are exposed on thrombasthenic platelets after stimulation (Mustard *et al.* 1979) despite the normal binding of [^{14}C]ADP (Legrand & Caen 1976) to the thrombasthenic platelet membrane. Thus one possible explanation for the absence of aggregation in thrombasthenia is that the fibrinogen bridge, which may link one platelet to another in the aggregate, is unable to form.

The IgG L... is an alloantibody isolated from the serum of a thrombasthenic patient; it induces a 'thrombasthenia-like' functional state on normal human platelets (Levy-Toledano *et al.* 1978). When the antibody was used in crossed immunoelectrophoresis it precipitated GPIIb/IIIa (Hagen *et al.* 1980), which appear to be present as a divalent cation dependent complex in Triton X-100 extracts of normal human platelets (Kunicki *et al.* 1981*c*). Recent studies have shown that IgG L... inhibits the ADP-dependent binding of fibrinogen to the normal human platelet surface (Lee *et al.* 1981). As Ca^{2+} ions are an essential cofactor for platelet aggregation (Born & Cross 1964), a tempting hypothesis is that GPIIb/IIIa may contribute structurally to the fibrinogen receptor that mediates platelet aggregation. Alternatively, in the absence of GPIIb/IIIa it is possible that the fibrinogen receptor does not become exposed after platelet stimulation with ADP or other aggregating agents.

Platelet adhesion to microfibrils present within the subendothelial tissues exposed during vessel injury appears to depend on the presence of plasma von Willebrand factor (v.W.f.) (Legrand *et al.* 1980). Ristocetin-induced platelet agglutination appears to be mediated by the binding of v.W.f. to the platelet surface (Kao *et al.* 1979). Both ristocetin-induced platelet agglutination and adhesion of normal human platelets to subendothelium were inhibited by an antibody that developed in a patient with the Bernard–Soulier syndrome who had received multiple transfusions of normal human platelet concentrates to arrest an episode of bleeding (Tobelem *et al.* 1976). Indirect immunoprecipitation tests with the use of this antibody (IgGP...) and Nonidet P40-extracted surface antigens of normal human platelets showed that it interacted with an antigen of apparent relative molecular mass 150000 (Degos *et al.* 1977). The antigen was absent from B.–S. platelets and appeared to be GPIb. The effect of the IgGP... was specific; it did not inhibit ADP- or collagen-induced platelet aggregation. Recent studies have shown that B.–S. platelets lack the ristocetin-induced v.W.f. binding receptor on the platelet surface (Moake *et al.* 1980). A tempting hypothesis, therefore, is that GPIb may contribute structurally to the v.W.f. receptor that appears to mediate platelet adhesion to microfibrils. Alternatively, it is possible that in the absence of GPIb this receptor does not become exposed after platelet stimulation.

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Conclusions

Blood platelets provide the initial response to injury in normal vessels by forming a platelet plug to prevent bleeding and to promote vessel healing. Since most platelet functions are mediated through cell surface interactions, the study of platelet membrane glycoproteins has been an important area of research. Progress has been made in defining the functional roles of some of the surface constituents. Our approach has been to investigate the membrane defects present in the platelets of patients with inherited abnormalities of platelet function, and to examine the effects on platelet function of antisera monospecific for different membrane antigens. Such studies have suggested specific roles for different membrane glycoproteins in platelet aggregation and adhesion. It is not yet known whether membrane glycoproteins directly mediate these processes or that their absence in abnormal membranes prevents the exposure of receptors for protein cofactors that mediate the platelet response after platelet activation. Studies are continuing to identify those molecules responsible for platelet 'stickiness', both after normal platelet activation and in platelet pathology.

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Discussion

J. R. O'Brien (*Central Laboratory, St Mary's Hospital, Portsmouth, U.K.*). Professor Caen's two types of thrombasthenia seem quite distinct. Has he examined patients with a bleeding diathesis in whom aggregation is diminished but not absent? We have two such families and so I wonder if there may be other variants.

J. P. Caen. There are two types of thrombasthenia without any aggregation, but outside these two groups there is also a group of mild thrombasthenia to which the family Dr O'Brien mentions probably belongs.

It would be very interesting to look at the fibrinogen binding and at the glycoprotein content of those platelets.

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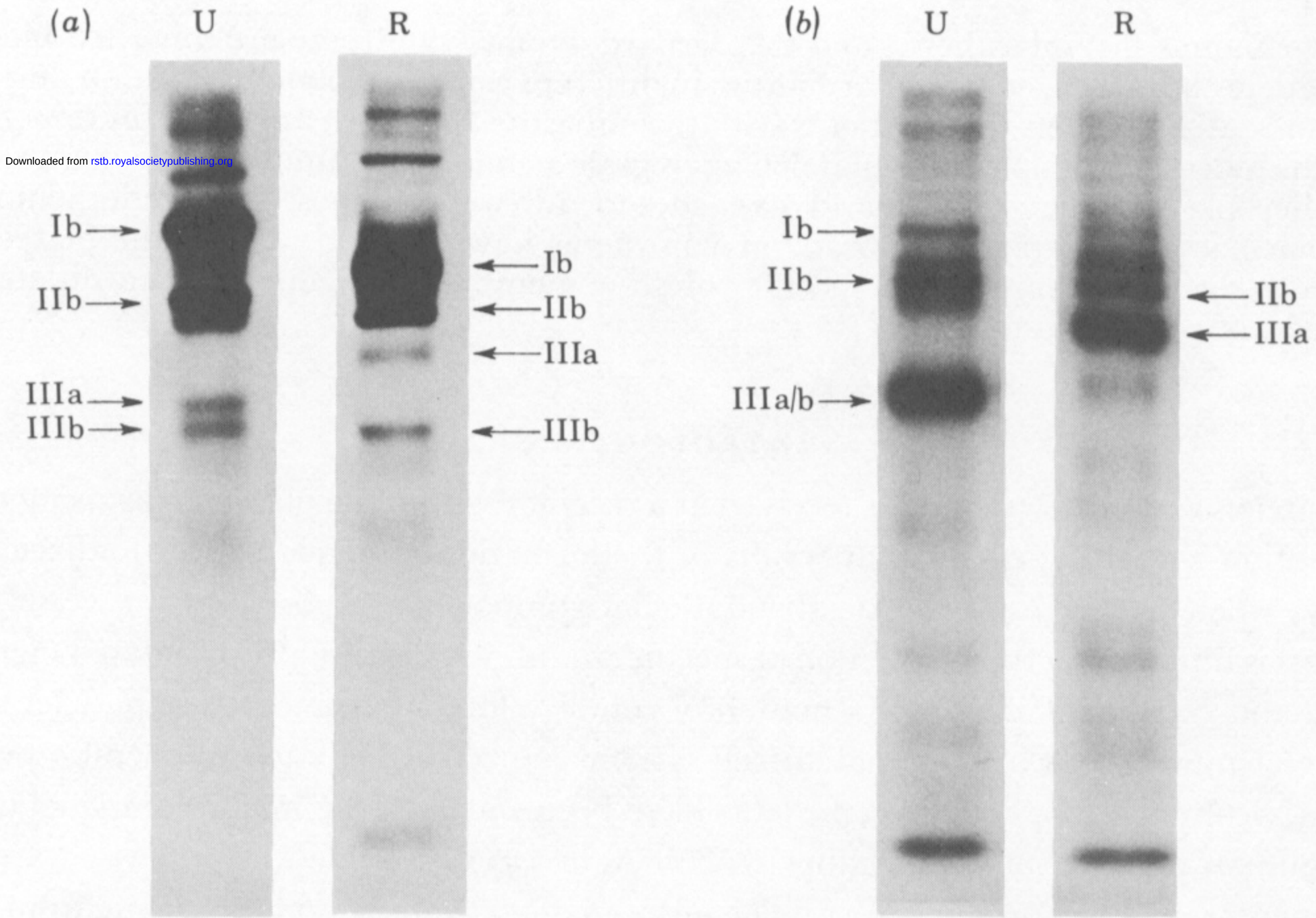

FIGURE 1. Identification of the surface-orientated membrane glycoproteins of normal human platelets after analysis by SDS–polyacrylamide gel electrophoresis of platelets whose surface proteins had been radiolabelled by two different surface labelling procedures. Washed human platelets were incubated (*a*) sequentially with neuraminidase, galactose oxidase and sodium [^{3}H]borohydride as described by McGregor *et al.* (1979), or (*b*) with lactoperoxidase and ^{125}I as described by Phillips & Poh Agin (1977*a*). The radiolabelled platelets were analysed by SDS–polyacrylamide gel electrophoresis on 7–12% gradient acrylamide slab gels in the absence of (unreduced; U) or after (reduced; R) disulphide bond reduction as detailed by Nurden *et al.* (1981). Radiolabelled proteins were located by fluorography (^{3}H) or by radioautography (^{125}I).

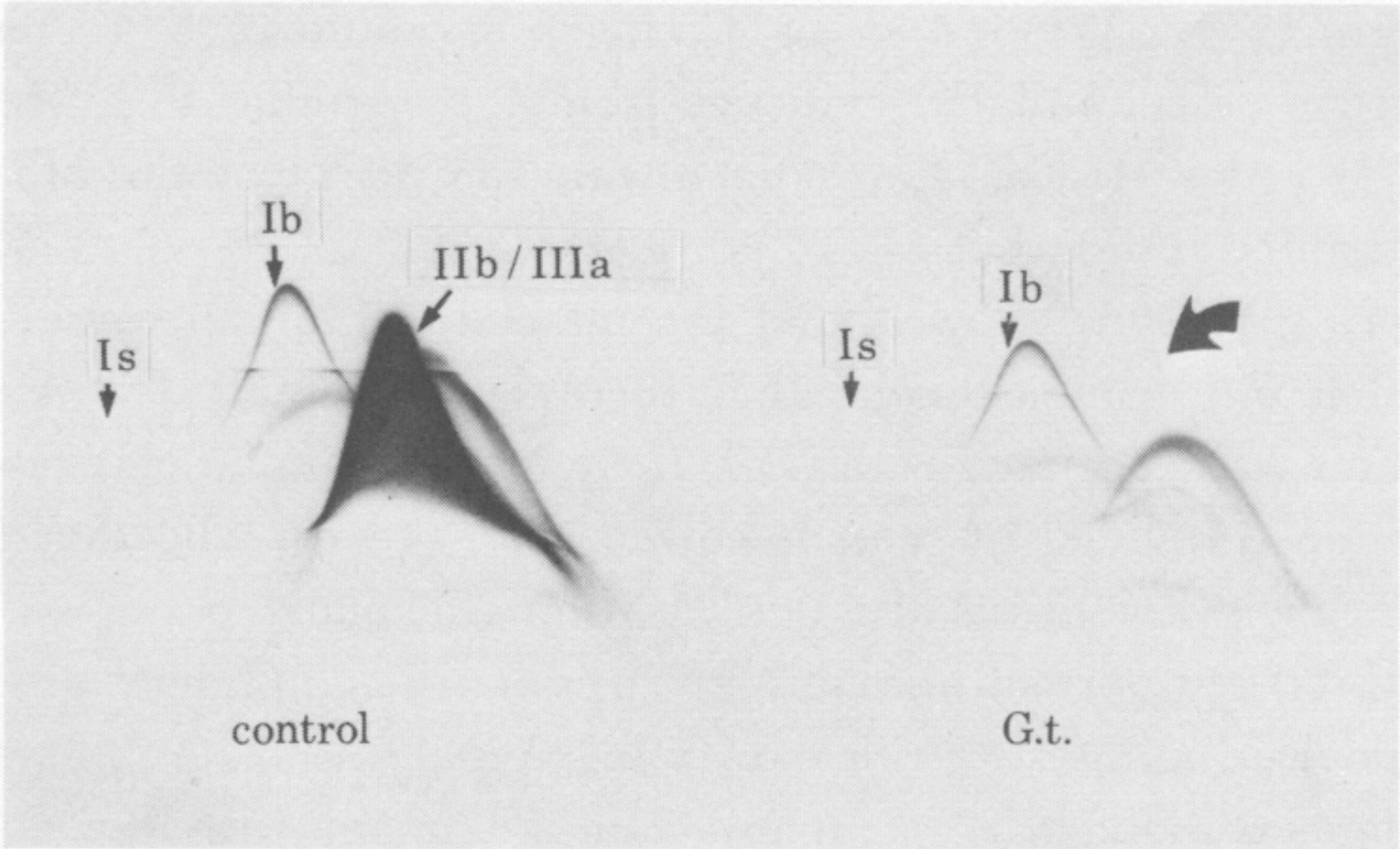

FIGURE 3. Analysis of the membrane glycoprotein composition of thrombasthenic platelets by crossed immunoelectrophoresis. The surface proteins of normal human platelets and those isolated from a patient with G.t. were labelled with ^{125}I by the lactoperoxidase-catalysed procedure. The platelets were solubilized in the presence of 1% Triton X-100 and c.i.e. performed according to the procedures detailed by Hagen *et al.* (1980). The immunoglobulin fraction of a multispecific rabbit antiserum prepared against washed, normal human platelets was incorporated into the agarose gel for the second dimension of electrophoresis. The immunoprecipitates containing radiolabelled antigens were located by radioautography.

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