Type I Glanzmann Thrombasthenia Patients From the Iraqi-Jewish and Arab Populations in Israel Can Be Differentiated by Platelet Glycoprotein IIa Immunoblot Analysis

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Glanzmann thrombasthenia is an autosomal recessive disorder of platelet function, characterized by excessive hemorrhage from mucocutaneous sites. Laboratory features include decreased or absent platelet functions, including clot retraction; adhesion to glass; and platelet aggregation in response to ADP, epinephrine, and thrombin. Affected platelets also fail to interact normally with both fibrinogen and von Willebrand factor (vWF) when stimulated with ADP or thrombin. The discovery of a deficiency of the glycoprotein IIb/IIIa complex in thrombocytopenic platelets provided a molecular basis for the disease, since there is evidence that this complex can function as a receptor for fibrinogen and fibronectin. Patients who meet the minimal clinical and laboratory criteria for Glanzmann thrombasthenia (a lifelong bleeding diathesis associated with absent platelet aggregation in response to adenosine diphosphate [ADP] and decreased clot retraction), have, however, displayed considerable heterogeneity in clinical symptoms and laboratory data, leading to attempts to categorize the disease into different types. Patients with type I Glanzmann thrombasthenia are said to have absent clot retraction and little or no platelet fibrinogen, whereas patients with type II are said to have some clot retraction and readily detectable, if somewhat reduced, platelet fibrinogen. By crossed immunoelectrophoresis and electroimmunoassay, several patients with the laboratory criteria for type I thrombasthenia had no detectable GPIIb/IIIa complex, whereas several patients who met the criteria for type II had reduced but detectable levels of the complex.

Early studies of the platelet glycoproteins in Glanzmann thrombasthenia patients utilizing polyacrylamide gel electrophoresis to separate the glycoproteins, which were either prelabeled before solubilization or detected with stains specific for carbohydrate and/or protein after electrophoresis, suggested that most patients shared the same abnormality, namely, a marked reduction but probably not absence of both GPIIb and GPIIIa. Subsequent studies indicated that there was more heterogeneity in glycoprotein patterns, with some patients having no detectable GPIIb and GPIIIa, others having discordant amounts of the two glycoproteins, and others having considerable amounts of both glycoproteins. Most recently, Norden studied seven type I and two type II thrombocytopenic patients with the technique of electrophoresis with antibodies separated by polyacrylamide gel electrophoresis onto nitrocellulose paper followed by immunologic detection of the glycoproteins with specific heterologous antisera. As expected, the type II patients had readily detectable GPIIb and GPIIIa. The type I patients, however, had unexpected heterogeneity, with only one demonstrating the absence of both GPIIb and GPIIIa. These data emphasized the rarity of the complete absence of both glycoproteins, even in type I patients, and highlighted the insensitivity of the crossed immunoelectrophoresis technique for quantifying the individual glycoproteins.

We recently reported the immunologic and biochemical characterization of the platelets of 32 patients from 19 families with thrombasthenia from the Iraqi-Jewish and Arab populations in Israel. All 19 patients whose platelets were analyzed by polyacrylamide gel electrophoresis were devoid of GPIIb, as judged by carbohydrate and protein staining. Patient platelets bound only ~2% of the normal amount of a monoclonal antibody (MoAb; 10E5) directed against GPIIb and/or GPIIIa; this antibody reacts with the glycoproteins when they are complexed with another but not

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when the complex is dissociated with calcium chelating agents. In fact, it was unclear whether this minimal amount of antibody binding represented a significant increase above background. GPIIIa could not be assessed in that study because platelet samples for polyacrylamide gel electrophoresis were analyzed in the nonreduced state, and under this condition another glycoprotein (GPIIb) comigrates with GPIIIa. In the present study we have analyzed 15 patients from 11 families in the Iraqi-Jewish population and four patients from four families in the Arab population with an immunoblot technique using a heterologous antibody to GPIIIa and a highly sensitive immunogold/silver enhancement detection technique. Our studies demonstrate one major immunoreactive band that appears to be related to GPIIIa and three minor bands that may be related to GPIIIa. In all 15 Iraqi-Jewish patients, the major GPIIIa band was not detectable. In 14 of the 15 patients the minor bands were either reduced or absent, whereas in one patient two of the minor bands were more prominent. In sharp contrast, all four Arab patients had reduced but detectable levels of the major GPIIIa band, reduced or absent minor bands, and an additional band of lower molecular weight (mol wt). We conclude that different protein and thus presumably genetic abnormalities produce the thromboclastic syndrome in the Iraqi-Jewish and Arab populations in Israel even though the patients in both groups meet the traditional criteria for type I disease.

MATERIALS AND METHODS

Patients. The patients investigated in this study come from the populations previously described. In all 15 Iraqi-Jewish patients from 11 families and four Arab patients, each from a different family, were studied.

Platelet preparation. Platelets from citrated blood were washed as previously described. Samples were stored frozen at −20°C.

Platelet preparation. Platelets from citrated blood were washed as previously described. Samples were stored frozen at −20°C. Control platelets samples were obtained from normal individuals both in Israel and at Stony Brook; the former were prepared earlier, at the same time as the patient samples, whereas the latter were prepared later, within weeks of when the samples were assayed by the immunoblot technique. No differences were observed between these different controls.

Antibodies

Rabbit anti-GPIIIa serum. GPIIIa was purified from human platelets by a modification of the differential NaCl extraction technique of Jennings and Phillips and used to immunize a rabbit as follows. Six units of outdated platelet concentrates (kindly provided by Dr Dennis Galanakis of the blood bank at the State University of New York at Stony Brook Hospital) were pooled, made 1 mmol/L in EDTA, and washed two times with 0.15 mol/L NaCl, 0.01 mol/L Tris, 1 mmol/L EDTA, pH 7.4. The platelets were divided in half and pelleted separately. One pellet (A) was resuspended in 25 mL of the above buffer and the other (B) was resuspended in 25 mL of 0.15 mol/L NaCl, 0.01 mol/L Tris, 1 mmol/L CaCl₂, pH 7.4. The suspensions were kept on ice, and Triton X-100 was added to bring the final concentration to 0.1%. After 55 minutes, the suspensions were centrifuged at 80,000 g for 60 minutes at 4°C. The pellets were brought up in 10 mL of the above buffers, but in each case the NaCl was omitted. The suspensions were again made 0.1% in Triton X-100 and allowed to incubate on ice for 60 minutes. The samples were then recentrifuged at 80,000 g for 60 minutes at 4°C. The supernatants, which contained approximately the same amount of both GPIb and GPIIIa as judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), were then chromatographed individually on a 0.9 × 25 cm column of Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) with an elution buffer of 0.05 mol/L sodium phosphate, 0.05% Triton X-100, and 0.05% sodium azide. The fractions from both columns that contained the GPIb/IIIa complex were pooled, made to contain 10 mol/L EDTA and 0.2% SDS in an attempt to dissociate GPIb from GPIIIa, and then rechromatographed on the Sephacryl S-300 column with an elution buffer (0.15 mol/L NaCl, 0.01 mol/L Tris, 10 mmol/L EDTA, 0.2% SDS, pH 7.4) designed to maintain the separation of GPIb from GPIIIa. The fractions containing purified GPIIIa, as judged by the presence of a single band by silver staining after SDS-PAGE, were pooled, and subsamples were removed for digestion by pepsin. First, 0.11 volume of 2 mol/L NaCl, 2 mol/L Na acetate, pH 4.0 was added to the sample (2 mL), and then 22 µL of pepsin A (1 mg/mL in 0.2 mol/L NaCl, 0.2 mol/L Na acetate, pH 4; Worthington Biochemical Co, Freehold, NJ) was added, bringing the final concentration to 10 µg/mL. The digest was kept at 37°C, and additional aliquots of pepsin were added at 1.3 hours, 2.3 hours, 17.6 hours, and 25.3 hours. After digestion, intact GPIIIa could be detected by SDS-PAGE. A mixture consisting of 1 mL of undigested GPIIIa and 0.75 mL of pepsin-digested GPIIIa was then emulsified with an equal volume of complete Freund’s adjuvant (Sigma Chemical, St. Louis). The back of an albino rabbit was shaved, and 0.1 mL of the emulsion was injected intradermally at multiple sites via a 25-g scalp infusion set with the aid of a syringe infusion pump (model 975, Harvard Apparatus, Millis, MA). The rabbit was re inoculated on day 11, day 21, and day 71. On day 80, the animal was anesthetized, and approximately 120 mL of blood was obtained via cardiac puncture. The blood was allowed to clot at 37°C for three hours in a glass tube, after which the serum was decanted, centrifuged at 2,000 g for ten minutes at 22°C to remove residual erythrocytes, and the supernatant serum heat-inactivated at 60°C for 20 minutes before being aliquotted and frozen at −20°C.

Murine monoclonal anti-GPIIIa antibody. This antibody, designated 16, was kindly supplied by Dr Simon Karpatkin of New York University School of Medicine. The antigen was partially purified from washed platelets by selective detergent solubilization in Triton X-114 followed by final purification by SDS-PAGE. The portion of the gel containing the GPIIIa band was excised and the protein eluted from the gel; it was then used to immunize a mouse. The latter’s splenocytes were fused to a mouse myeloma cell line by conventional methods and the supernatants assayed for reactivity with GPIIIa. For the present studies, culture supernatants were precipitated with 50% ammonium sulfate, after which the precipitates were dissolved in one-fourth the original volume of phosphate-buffered saline (PBS) containing 0.025% sodium azide and then dialyzed, first against water and then against the same phosphate buffer, before being frozen.

Human anti-PF IV antibody. This antibody (also kindly supplied by Dr Simon Karpatkin) was from a male patient who developed thrombocytopenia after cardiac surgery as a result of post-transfusion purpura. The patient’s serum was found to contain an antibody (or antibodies) reactive with the PF IV antigen, which is known to reside on GPIIIa. The patient’s plasma was obtained by plasmapheresis and a fourfold concentrated immunoglobulin fraction was processed as described above for the MoAb.

SDS-PAGE. Solubilized platelets were mixed 2:1 with a sample buffer containing 3.05% SDS, 0.18 mol/L Tris/Cl, 30% glycerol,
and 0.02% bromophenol blue, after which they were heated to 100°C for three minutes. A 38-μL sample containing ~150 μg platelet protein was then applied to a 1.5-mm thick gel containing a 1 × 10-cm 3% acrylamide stacking gel and a 5.4 × 10-cm 7% resolving gel containing the discontinuous buffer system of Laemmli. The running buffer was 0.05 mol/L Tris/glycine, 0.4% SDS, pH 8.9. Electrophoresis was carried out at 50 V until the sample entered the separation gel (Mighty Small slab gel electrophoresis unit, SE 200, Hoefer Scientific Instruments, San Francisco) (~30 minutes); the voltage was then increased to 150 V and electrophoresis proceeded until the blue tracking dye approached the bottom of the gel. Earlier samples were electrophoresed at 150 V, but this was found to result in broadening of the protein bands. When similar gels were stained with a silver reagent (Bio-Rad silver stain kit, Bio-Rad Laboratories, Richmond, CA), which only stains the protein on the outer surfaces of the gel, it was found that the protein on the gel surface closer to the cooling plate migrated less rapidly than the protein on the gel surface exposed to the ambient air. Thus the broadening was presumed to reflect differential thermal effects, and this was minimized by decreasing the voltage to 100 V and heating the running buffer to 37°C. Proteins of known mol wt (Sigma Chemical Co., St. Louis) were also electrophoresed on each gel (carbonic anhydrase 29,000; egg ovalbumin, 45,000; bovine plasma albumin, 66,000; rabbit muscle phosphorylase B, 94,000; Escherichia coli β-galactosidase, 116,000; and rabbit muscle myosin, 250,000).

**Immunoblotting.** After electrophoresis was finished, the lane containing the mol wt standards was cut from the gel and stained with Coomassie blue; the proteins contained within the remainder of the gel were transferred to nitrocellulose paper (0.2 μ pore size; Schleicher and Schuell, Keene, NH) using an electrophoresis apparatus (Mini Transphor; TE22; Hoefer Scientific Instruments) according to a modification of the technique described by Towbin et al. In particular, two pieces of paper were placed anodally to the gel, and one was placed cathodally; only the first piece placed anodally was used for immunoblotting, whereas the others were washed in PBS pH 7.2 containing 0.3% Tween 20 and then stained with a colloidal gold reagent (AuroDye, Janssen Life Sciences Products, Beenge, Belgium) to assess the completeness of the transfer. The transfer buffer (0.192 mol/L glycine, 25 mmol/L Tris, pH 8.3 containing 20% [vol/vol] methanol) was thoroughly degassed and cooled to 4°C before use; electrophoresis was carried out with tap water cooling (~18°C) at 400 m Amps for one hour. Coomassie blue staining of the gel after transfer showed that there was nearly complete loss of proteins below mol wt 180,000 and partial loss of higher mol wt proteins.

The immunoreactive bands on the nitrocellulose paper were then detected by reaction with a gold-conjugated anti-immunoglobulin antibody and subsequent enhancement of the gold stain with a silver reagent (AuroProbe BL IGSS kit; Janssen). The nitrocellulose was incubated with a blocking solution (20 mmol/L Tris/Cl, 0.9% NaCl, 5% bovine serum albumin [BSA], 20 mmol/L sodium azide, pH 8.2) for two hours at 37°C and then overnight at 4°C, after which it was washed with the same Tris-NaCl buffer containing 0.1% BSA. The paper was then incubated with gentle shaking for two hours at 22°C with dilutions of the human anti-P1 antibodies (mol wt -180,000) or the monoclonal mouse anti-GPIIIa immunoglobulin fraction (1/100), the monoclonal mouse anti-GPIIIa immunoglobulin fraction (1/2,500), or the rabbit anti-GPIIIa serum (1/50) in the Tris-NaCl, 0.1% BSA, pH 8.2 buffer to which 1% normal goat serum was added. After washing in Tris-NaCl, 0.1% BSA buffer three times, the paper was incubated with gentle shaking with 1/100 dilutions of affinity-purified goat antibodies to human, mouse, or rabbit immunoglobulins that were conjugated to 20 mmol/L colloidal gold particles. The antibodies were diluted in Tris-NaCl, pH 8.2 buffer containing 0.4% gelatin (wt/vol) and 20 mm sodium azide and the incubation proceeded overnight at 22°C. Light pink bands could be detected at this point. After washing with the Tris-NaCl, 0.1% BSA buffer, the intensity of the bands was enhanced by reaction with a freshly prepared silver stain (IntenSE, Janssen) that precipitates metallic silver on the gold surface and converts the pink bands to a gray-black color. Equal volumes of the enhancer (4.7 g/100 mL) and initiator (7.8 g/100 mL) solutions were mixed immediately before use and the nitrocellulose paper was stained for approximately ten minutes at 22°C. The paper was then placed in the fixer for five to ten minutes, after which it was washed in water and dried. The silver enhancement step led to some variability in the intensity of individual bands between blots, and so the current experiments were performed such that the control and patient samples were included on each blot, and each blot was developed as a unit.

**Characterization of the rabbit antiserum to GPIIIa.** The antiserum gave only a single arc against normal platelets when tested in radioimmunoelectrophoresis as judged by Coomassie blue staining. This arc was identified as containing the GPIIb/IIIa complex, since the radioautogram of the same gel showed unique incorporation of radiolabeled 10E5 into this arc, and no arc was present by either detection method when a sample of platelets from a patient with Glanzmann thrombasthenia was tested.

**Comparison of rabbit anti-GPIIIa, human anti-P1, and mouse monoclonal anti-GPIIIa antibodies.** All three antibody preparations recognized three proteins in nonreduced platelet samples, and these are designated 1, 2, and 3 in Fig 1. Band 2 was much more intense than the other two bands and corresponded in mol wt to the nonreduced value of purified GPIIIa (mol wt ~90,000). Studies using the rabbit

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**RESULTS**

**Immunoblot analysis of normal platelet proteins reacted with normal rabbit serum (NI Ser [R]), a human anti-P1 immunoglobulin fraction (α-P1 [H]), a mouse MoAb to GPIIIa (α-IIIa [Mm]), and a rabbit anti-GPIIIa antibody (α-IIIa [R]).** The mol wt of the reference proteins are indicated on the left (in kilodaltons), and the four immunoreactive bands are indicated on the right.

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antiserum and dilutions of the control platelet sample showed that this band could be detected at a dilution of 1/1,000, indicating a sensitivity in excess of 0.1%. Band 1 was of mol wt ~110,000, and band 3, which could not always be resolved from band 2, was of mol wt ~81,000. An additional band of mol wt ~64,000 was recognized by the rabbit anti-GPIIIa serum, but not by the other two immunoglobulin preparations. A variety of higher mol wt bands were also visible with both the rabbit anti-GPIIIa and human anti-Pl antibodies. One of these bands (mol wt ~160,000) was visible with the mouse monoclonal anti-GPIIIa antibody and to a variable extent with normal rabbit serum, and so it was believed to represent cross reactivity of the second antibodies with the human IgG present in the platelet preparations. Some of the other high mol wt bands and some bands in the mol wt 80,000 to 90,000 range were also visible to a lesser extent when normal rabbit serum was used instead of the immune serum, and so they were not considered specific. When platelet samples were reduced with 5% β-mercaptoethanol before SDS-PAGE, no bands were detected with any of the three different immunoglobulin preparations. This probably results from a marked change in the tertiary structure of this cysteine-rich protein, which also undergoes a large increase in mol wt with reduction.

Comparison of immunoblot patterns of platelet proteins from Iraqi-Jewish and Arab patients with thrombasthenia. The immunoblots of control and patient platelets reacted with the rabbit anti-GPIIIa serum are shown in Fig 2. The platelet sample from controls 1, 5, and 6 were prepared at Stony Brook, whereas those from controls 2, 3, 4, 7, and 8 were prepared at the National Blood Center.
and 4 were prepared in Israel. All of the controls had the same pattern as that described above, although band 3 was resolved to variable degrees. The samples from all four Arab patients gave a pattern that was distinctly different from the control, but very similar with one another. Band 1 was reduced in intensity and more diffuse, being nearly undetectable on some blots but not others (eg, A-I in panel A of Fig 2 v A-I in panel D); band 2 was markedly reduced in intensity but still easily visible; and bands 3 and 4 were markedly reduced, being barely visible. Moreover, there appeared to be an additional or enhanced band at mol wt ~47,000 in all four Arab samples.

In contrast to the Arab samples, band 2—the intensely staining mol wt-90,000 protein—could not be detected in the platelet samples obtained from any of the 15 Iraqi-Jewish patients tested. Band 4 also seemed to be missing from all but one (I-J 9) of the Iraqi-Jewish samples. The other two minor bands were either reduced or absent in the platelets from these 14 patients, but the extent of their deficiency was hard to judge because of the variable intensity of nonspecific bands (ie, bands that were also seen with normal rabbit serum) in these regions. One patient (I-J 9), who was unrelated to the other Iraqi-Jewish patients and whose last platelet transfusion was ~1 week before this sample was obtained, had a unique pattern consisting of a reduced but clearly detectable band 1; the absence of both band 2 and band 3; and a slightly reduced but still detectable band 4. This same pattern was obtained when this patient’s platelet sample was analyzed a second time and with a second sample obtained ~1 month after a platelet transfusion.

**DISCUSSION**

A prolonged bleeding time, normal platelet count, absence of adhesion to glass, and absence of the primary wave of ADP-induced platelet aggregation are some of the hallmarks of Glanzmann thrombasthenia. The uniformity of these findings, however, belies the extraordinary clinical heterogeneity of the disorder. Although subtle variability even in the hallmark criteria were recognized in early studies, more dramatic variability was identified in the deficiencies in clot retraction, platelet fibrinogen content, and fibrinogen binding to ADP-activated platelets. This led to attempts to categorize the disorder as type I (absent clot retraction, marked fibrinogen deficiency, and virtually absent fibrinogen binding) or type II (decreased clot retraction, mild or no fibrinogen deficiency, and normal or only slightly reduced fibrinogen binding), but it was not clear whether there was a correlation between the clinical symptoms and these laboratory findings.

The discovery that thrombasthenic platelets are deficient in glycoproteins IIb and IIIa and that these glycoproteins exist as a complex to which fibrinogen binds when platelets are activated, allowed for more detailed biochemical analysis of the platelet defects. A variety of techniques have been applied to separating and identifying these platelet proteins, including ones that utilize antibodies directed against either the whole proteins or against PI, an antigen localized to GPIIa. Monoclonal antibody binding to intact platelets has also been employed. These techniques were able to identify a subpopulation of patients who had reduced but still readily detectable levels of GPIIb and GPIIIa; quantitative estimates suggested that such patients had anywhere from a trace amount up to 50% of the normal amount of the glycoproteins. The laboratory data from several such patients indicated a type II pattern, but other patients were found to be discordant with regard to the type II criteria. For example, individual patients have been described who have (1) a severe deficiency of platelet fibrinogen despite having considerable amounts of residual GPIIb and GPIIIa, (2) considerable blood clot retraction despite markedly reduced platelet fibrinogen and undetectable amounts of the GPIIb/IIIa complex, and (3) nearly normal amounts of platelet fibrinogen despite having markedly reduced or undetectable levels of GPIIb and GPIIIa. The group designated originally as type II is markedly heterogeneous at the biochemical level, and this complexity is compounded by the discovery of unusual variant patients who have many of the laboratory and clinical manifestations of Glanzmann thrombasthenia but normal or nearly normal amounts of GPIIb and GPIIIa.

The patients who meet the laboratory criteria for type I thrombasthenia also appear to be heterogeneous at the biochemical level. Considerable confusion surrounds whether or not such patients have totally absent or only markedly reduced levels of the glycoproteins. This is not surprising given that different investigators studied different patients and that the methods used to detect the glycoproteins differ considerably in sensitivity. It is extremely important to determine whether the platelets of such patients have even small amounts of the glycoproteins, since this has important implications for the genetics and cellular biology of the disorder. Among the gel electrophoresis techniques, the nonreduced-reduced and two-dimensional gel techniques provide more precise localization of given glycoproteins than does the one-dimensional technique and thus overcome the problem of overlapping proteins to a large extent. Since they are standardized with normal proteins, however, they may not be able to detect abnormal forms of the protein that migrate to other positions. Radioiodination clearly increases the sensitivity of detection when compared to staining techniques, but such studies need to be interpreted in the light of the labeling’s dependence on specific chemical structure that may be abnormal in the disease states. In particular, since some thrombasthenic patients may have a generalized abnormality in the attachment of carbohydrates to platelet glycoproteins, techniques that label carbohydrate structures may be misleading. Moreover, most labeling studies are performed with intact platelets using membrane-impermeant reagents so that only the surface glycoproteins are labeled. Such techniques would not detect abnormal glycoproteins that cannot insert into the plasma membrane, as has been reported to occur in at least one patient with thrombasthenia.

The agarose gel immunologic techniques that employ heterologous antisera have focused on the arc corresponding to the glycoprotein IIb/IIIa complex; the absence of either glycoprotein would therefore result in the absence of this arc,
regardless of how much of the other glycoprotein was produced. Although coordinate synthesis of GPIIb and GPIIIa has been assumed to occur under normal conditions, data suggest that in some patients with thrombasthenia the synthesis and/or surface expression may not be coordinate. In some patients with thrombasthenia the synthesis and/or surface expression may not be coordinate.22-24,27 Thus the absence of a GPIIb/IIIa complex arc cannot be taken as evidence for the absence of both glycoproteins. The binding of radiolabeled MoAbs to intact platelets28-48 must be interpreted not only with regard to the specificity of the antibody and the surface expression of the glycoprotein, but also the ability to determine whether a given low level of binding to platelets is significantly increased above background.

The immunoblot technique, combining the resolution of SDS-PAGE and the detection capabilities of polyclonal antibodies to purified GPIIIa is probably the most sensitive technique currently available; it does, however, depend on the antigenicity surviving solubilization in SDS as well as the abnormal protein sharing at least some immunologic determinants with the normal protein. Assuming that SDS solubilizes essentially all of the platelet proteins, it has the added advantage of measuring the total amount of the glycoproteins, not just that present on the platelet surface or that which can be solubilized in Triton X-100. Nurden et al27 studied seven unrelated patients who met the laboratory criteria for type I thrombasthenia and two patients who met the criteria for type II with this technique, using radiolabeled protein A to detect the immunoreactive bands. They estimated the sensitivity of their immunoblots at 1% for GPIIb and 0.5% for GPIIIa. The two type II patients, who previously were shown to have 13% and 15% of normal GPIIb/IIIa complex by crossed-immunoelectrophoreses, had GPIIb and GPIIIa bands that were only moderately decreased in intensity when compared to normal. The type I patients were heterogeneous: three patients had weak GPIIb bands and strong GPIIIa bands; three patients had no detectable GPIIb bands and reduced but detectable GPIIIa bands; and in only one patient was there no detectable GPIIb and GPIIIa.

In the present study we have applied a modified immunoblot technique for GPIIIa to the thrombasthenic patients in Israel who come primarily from the Iraqi-Jewish and Arab populations. All of these patients were studied previously and found to have laboratory evidence of type I thrombasthenia.28,31

Our antibody to GPIIIa identified a major band at mol wt 90,000, which corresponds in mol wt to purified GPIIIa. In addition, however, there were minor bands at mol wt ~110,000, 81,000, and 64,000. The mol wt 110,000 and 81,000 bands were also identified by a human antibody to the PIα antigen (known to be present on GPIIIa)35 and a murine MoAb to GPIIIa, suggesting that they are related to GPIIIa rather than being due to contamination of the original antigen with additional platelet proteins.

After the present studies were completed, Dancis et al independently reported on the characterization of the monoclonal anti-GPIIIa and the human anti-PIα antibodies used in this study.34 They found that both antibodies reacted in immunoblots of normal platelet membranes with a major band at mol wt 100,000 (comparable to our band 2, mol wt 90,000), a band 22,000 higher in mol wt (comparable to our band 1, mol wt 110,000), and a lower band of mol wt 71,000 (comparable to our band 4, mol wt 64,000). They further showed that these bands are immunologically related because a similar MoAb lost reactivity against all three bands when adsorbed with the major band and that an affinity column containing the MoAb used in this study immunopurified both the mol wt 100,000 and mol wt 122,000 proteins. They did not identify a band comparable to our band 3, but this may be related to minor technical differences, since we found that this band was difficult to resolve from the heavy, and broad band 2. It is not clear why they were able to identify the mol wt-71,000 band with these antibodies, whereas we were only able to identify a similar band with our heterologous antisera. If this band is a proteolytic fragment of GPIIIa, it is possible that this is a matter of sensitivity, since they used platelet membrane preparations that may have been more likely to have undergone proteolysis during preparation than the whole platelet samples that we employed. In fact, chymotrypsin digestion of GPIIIa has been reported to result in the production of fragments of mol wt 58,000 to 60,000 that react with anti-Plα sera22,32,33 and may be comparable to these lower mol wt bands. It will remain for more detailed biochemical analyses of these minor bands to establish their relationship, if any, with GPIIIa. Interestingly, the minor bands observed by Dancis et al34 and in the present study have not been reported before in immunoblotting studies of GPIIIa using either anti-GPIIIa or anti-PIα sera.27,35,36 The technique we used, which employs a gold-labeled second antibody and a subsequent silver enhancing step, is said by the manufacturer to be more sensitive than other methods, and this may account for these minor bands being detectable. In fact, we assessed the sensitivity for GPIIIa to be at least fivelord greater than that reported by Nurden.19

It is tempting to speculate that the mol wt-110,000 band is a precursor of GPIIIa, but, in fact, post-translational changes in carbohydrate (or other moieties) or modifications in the disulfide bond formation may account for the higher apparent mol wt. Moreover, Kieffer et al35 found that a human anti-Plα serum that reacted only with a mol wt-89,000 GPIIIa band on native platelets reacted with a mol wt-105,000 protein when platelets were treated with chymotrypsin; they speculated that the limited proteolysis may have altered the conformation of the molecule in a manner similar to that produced by disulfide bond reduction, which is known to increase the apparent mol wt of GPIIIa in SDS-PAGE. Thus if a low level of endogenous protease activity exists, perhaps a minor component of higher apparent mol wt could be generated.

Our studies were aided by having a large number of patients from the Iraqi-Jewish population in which generations of intramariage made it likely that most patients share the same genetic defect. This allowed us to analyze the consistency of immunoblot patterns in such a population. In fact, 14 of the 15 patients studied from this population had the same immunoblot pattern: no detectable major band and either reduced or absent minor bands. Thus none of the techniques used to study these patients have been able to
clearly identify any residual GPIIb or GPIIIa, leaving open the possibility that these patients suffer from a deletion of genetic information.

The one Iraqi-Jewish patient who differed from the rest of this group had a unique pattern: absent mol wt-90,000 and mol wt-81,000 bands and reduced but clearly detectable mol wt-110,000 and mol wt-64,000 bands. Although it is possible to propose models that might account for such a finding and still be consistent with all of the protein bands being related to GPIIIa, more information is required before a plausible model can be proposed. Since his last platelet transfusion was ~1 month before one of the samples was obtained, it is unlikely that any of the transfused platelets remained in his circulation. The presence of only the mol wt-110,000 and mol wt-64,000 bands argues against these bands being the result of minor in vitro proteolysis of the mol wt-90,000 band. It is interesting to speculate on the genetics in this patient and how they may differ from the genetics in the other Iraqi-Jewish patients. In particular, since the frequency of the carrier state of the commoner defect is so high in this population, it is possible that this patient is doubly heterozygous for two different defects rather than homozygous for a different recessive gene.

The four Arab patients come from four unrelated families currently living in different regions in Israel. It was interesting, therefore, that all four patients tested had the same pattern: reduced but detectable major band, reduced minor bands, and the appearance of a new band at mol wt 47,000. Thus these patients cannot have a total deletion of GPIIIa genetic information. Moreover, given the heterozygosity previously observed in immunoblot studies of type 1 thrombasthenics, the similarity in patterns in all four patients suggests that they may share the same genetic defect. Although there are many different ways to explain the presence of the additional band, one simple interpretation is that the patients make an abnormal form of the protein that is cleaved by a protease(s) at this site.

The consistency of the immunoblot patterns in Iraqi-Jewish and Arab populations emphasizes the relationship among members of each group and their difference from patients in the other group. In this way it demonstrates the power of the technique to differentiate among patient populations that are identical by all other methods. As DNA probes for these glycoproteins become available, it will be interesting to correlate the immunoblot data with the DNA abnormalities.

NOTE ADDED IN PROOF
Since submission of this manuscript, we have studied another Arab patient with thrombasthenia. The patient is now living in the United States, and the sample was kindly obtained by Dr David Green of Northwestern University (Chicago). As with the other Arab patients, the Mr 90,000 band was markedly reduced, but still readily detectable, indicating the presence of GPIIIa.

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Type I Glanzmann thrombasthenia patients from the Iraqi-Jewish and Arab populations in Israel can be differentiated by platelet glycoprotein IIa immunoblot analysis

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