

Diagnosis of Bernard-Soulier Syndrome and Glanzmann's Thrombasthenia with a Monoclonal Assay on Whole Blood

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ABSTRACT Two hereditary platelet disorders, Bernard-Soulier syndrome and Glanzmann's thrombasthenia, are characterized by selective deficiencies of platelet membrane glycoproteins. Murine monoclonal antibodies were developed against platelet membrane glycoprotein Ib and against the glycoprotein IIb/IIIa complex. A rapid whole blood assay for the deficiency of these glycoproteins was developed and used to study whole blood samples from six patients with Glanzmann's thrombasthenia and three patients with Bernard-Soulier syndrome. Patients with type I and type II Glanzmann's thrombasthenia were easily detectable with this assay. This permits the diagnosis of these disorders on 200 μ l of whole blood within 2 h of blood sampling.

INTRODUCTION

Certain hereditary platelet disorders have been characterized by a deficiency of specific platelet membrane glycoproteins. In one of these disorders, Bernard-Soulier syndrome, the platelets lack the platelet membrane glycoprotein (GP)¹ Ib (1-3), and in another, Glanzmann's thrombasthenia, there is a deficiency of GPIIb and GPIIIa (1-6). Recently, several laboratories have

developed monoclonal antibodies to these glycoproteins (7-10), and in one of these studies (7), thrombasthenic platelets were evaluated in a washed platelet assay by using a monoclonal antibody.

Although the diagnosis of Bernard-Soulier syndrome is suggested by giant platelets and an absence of ristocetin-induced platelet aggregation (1, 2, 11), the specific diagnosis is confirmed by detailed platelet membrane glycoprotein analysis (1, 2, 12). Unfortunately, Bernard-Soulier platelets are difficult to separate from whole blood; thus, the functional and glycoprotein analyses are not easily performed. In Glanzmann's thrombasthenia, studies demonstrate reduced or absent platelet aggregation, decreased clot retraction, a prolonged bleeding time (1), and reduced platelet GPIIb and GPIIIa (1, 2, 12, 13). Platelet membrane glycoprotein analysis is not a routine laboratory determination; thus, the majority of patients suspected of having these diagnoses may not have the glycoprotein deficiency confirmed.

In the process of preparing monoclonal antibodies to platelet membrane glycoproteins, we developed a rapid semiquantitative assay that can be performed on <1 ml of whole blood. Because whole blood is used, this obviates the preparation of platelet-rich plasma, which may be difficult to prepare from patients with Bernard-Soulier syndrome. In this report we describe the development of these monoclonal antibodies and their use in this diagnostic assay.

METHODS

Materials were obtained from the following sources: ristocetin from Lundbeck (Copenhagen) Denmark; Protein A-Sepharose from Pharmacia Fine Chemicals, Pharmacia, Inc.,

Dr. Montgomery is an Established Investigator of the American Heart Association, 82-198. Dr. Kunicki is the recipient of a New Investigator Award (HL 27638) from the National Heart, Lung, and Blood Institute. Address all correspondence to Dr. Montgomery.

Received for publication 24 September 1982 and in revised form 8 November 1982.

¹Abbreviations used in this paper: AP-1 and AP-2, antiplatelet 1 and 2, respectively; GP, glycoprotein.

(Piscataway, NJ); P3 X 63Ag8.653 myeloma cell line from Salk Institute (La Jolla, CA); Freund's complete adjuvant from Gibco Laboratories, Grand Island Biological Co. (Grand Island, NY); BALB/c mice from Simonsen (Gilroy, CA); BALB/c \times DBA/2 F₁ mice from Cumberland Farms (Clinton, TN); Na¹²⁵I from New England Nuclear (Boston, MA); Pristane and polyethylene glycol 1,500 from Aldrich Chemical Co., Inc. (Milwaukee, WI), Apiezon oil from James G. Biddle Co. (Blue Bell, PA); antibodies to specific heavy and light chains of mouse IgG from Bethesda Research Laboratories Inc., (Gaithersburg, MD); and all chemicals were reagent-grade (Sigma Chemical Co., St. Louis, MO).

Immunization of BALB/c mice. Pooled normal human platelets were washed as previously described (3). Erythrocyte and leukocyte contamination was $<1:2,000$ platelets. BALB/c female mice were given a primary intraperitoneal immunization with 1×10^9 washed platelets suspended in 0.5 ml of Freund's complete adjuvant. Three subsequent intraperitoneal immunizations with 1×10^9 washed platelets suspended in 0.9% saline were given at 2-wk intervals. 1 wk after the last injection, the mice were given 2×10^9 platelets intravenously. 4 d later the mice were killed and their spleen cells were used for fusion.

Hybridization, screening, and cloning. Hybridization was carried out with 2.5×10^8 of the above spleen cells mixed at a 1:1 ratio with P3 X 63Ag8.653 myeloma cells, according to the method of Oi and Herzenberg (14). Fusion was accomplished with 50% polyethylene glycol 1,500. Hypoxanthine-aminopterin-thymidine medium selection was carried out (14) and hybridized cells grown in hypoxanthine-thymidine medium for 2–3 wk. Positive hybridomas were selected by an enzyme-linked immunoabsorbent assay by using partially purified platelet membrane glycoproteins (GPIIb/IIIa complex and GPIb). In addition, a microtiter screening assay for the inhibition of ristocetin-induced platelet aggregation was developed. Each well contained 100 μ l of formalin-fixed platelets incubated for 2 h with 50 μ l of tissue culture fluid. Normal plasma (15 μ l) and 5 μ l of ristocetin (30 mg/ml) were added and the microtiter plate agitated overnight. The presence or absence of aggregation was noted. Absent aggregation suggested an antibody directed against GPIb. Positive hybrids were cloned by limiting cell dilution on feeder layers of peritoneal exudate cells (15).

Ascites tumors. Clones were grown to sufficient density and 5×10^5 cells injected intraperitoneally into "Pristane primed" (BALB/c \times DBA/2 F₁ mice. Mice were tapped for ascites fluid usually after 2–3 wk. Subsequent ascites tumors were produced from master cultures of the clones and not sequentially passed as ascites tumors.

Identification of specific monoclonal antibody to GPIb and GPIIb/IIIa. Monoclonal antibody was prepared from the ascites fluid by three sequential 50% ammonium sulfate precipitations, dialyzed against potassium phosphate buffer (0.01 M, pH 7.4), and applied to Protein A-Sepharose. Elution from the Protein A used Tris glycine buffer that was prepared by adding 2 M glycine HCl to 0.01 M Tris in order to bring the pH to 2.2. The pH of the eluted material was returned to neutrality (pH 7–7.5) with 1 M K₃HPO₄. The IgG was labeled with Na¹²⁵I by the chloramine T method, as described previously (16). Crossed immunoelectrophoresis was carried out against Triton X-100 solubilized platelet proteins, as previously described (12), except that labeled monoclonal antibody was incorporated into the second dimension along with the unlabeled rabbit antiplatelet antisera (17). Although the monoclonal antibodies were not precipitating, they bound to antigens contained in the precipitates

produced by the rabbit antisera, thereby substantiating the immunologic reactivity of the monoclonal antibodies.

Inhibition of ristocetin-induced platelet aggregation. The titer of antibody to GPIb was assessed by diluting the monoclonal antibody and incubating 10 μ l of this IgG with 400 μ l of formalin-fixed platelets before performing a ristocetin cofactor assay, as previously described (16).

Immunoglobulin class. Ouchterlony analysis of the monoclonal antibodies was carried out against specific anti-heavy chain and anti-light chain antisera.

Patient samples. Blood was drawn into routine acid citrate dextrose anticoagulant at a ratio of 1:6. Samples were obtained from 23 normal volunteers, 3 patients with Bernard-Soulier syndrome, and 6 patients with Glanzmann's thrombasthenia. The samples on the Bernard-Soulier syndrome patients were provided by Dr. Margaret Johnson, Wilmington, DE (two patients) and Dr. William Deardorff, Milwaukee, WI. The samples on the patients with Glanzmann's thrombasthenia were provided by Dr. Joan Gill (Milwaukee, WI), Dr. Margaret Johnson, Dr. David Green (Chicago, IL; two patients), and Dr. Juan Chediak (Chicago, IL; two patients).

Platelet counts were performed by phase-contrast microscopy, and all platelet counts were within the normal range except for the three Bernard-Soulier patients, who had platelet counts of 120,000, 78,000, and 65,000.

Whole blood monoclonal assay for platelet membrane glycoprotein deficiency. Whole blood (100 μ l) was diluted with 100 μ l of phosphate-buffered saline (0.05 M phosphate, 0.15 M NaCl, pH 7.4), and 10 μ l of radiolabeled monoclonal antibody (10^5 cpm) for 30 min at room temperature with constant gentle agitation. A 180- μ l sample was then layered over 500 μ l of Apiezon oil/N-butyl phthalate (1:9) and centrifuged at 11,000 g for 10 min. The aqueous layer, oil layer, and pellet were separately counted and the ¹²⁵I radioactivity of the bound (pellet) fraction determined. All experiments were done in duplicate or quadruplicate. Non-specific trapping of radiolabeled antibody was $<1\%$.

Quantitative anti-platelet (AP) 1 and 2 binding to platelets with platelet-rich plasma. Platelet-rich plasma (250,000 platelets/mm²) was prepared from two of the Bernard-Soulier patients and one of the thrombasthenic patients. Serial dilutions 1:2 to 1:512 were performed and the above monoclonal assay method repeated. Each dilution was tested in duplicate and compared with a normal platelet-rich plasma control with the same number of platelets.

RESULTS

Monoclonal antibody. Initially, eight hybridomas had antibodies that reacted with the partially-purified platelet glycoproteins. Clones specific for GPIb and GPIIb/IIIa were identified, cultured, and used to establish murine ascites tumors. This report concerns the first two clones, designated AP-1 and AP-2 by the order of their development. Sequential 50% ammonium sulfate precipitation of the ascites fluid followed by binding, then elution from Protein A, gave material that was essentially homogeneous IgG on polyacrylamide gel electrophoresis.

Characterization and specificity. AP-1 and AP-2 were both determined to have G₁ heavy chains and κ light chains by Ouchterlony analysis. AP-1 and AP-2 were radiolabeled with Na¹²⁵I (2×10^5 cpm/ μ g of

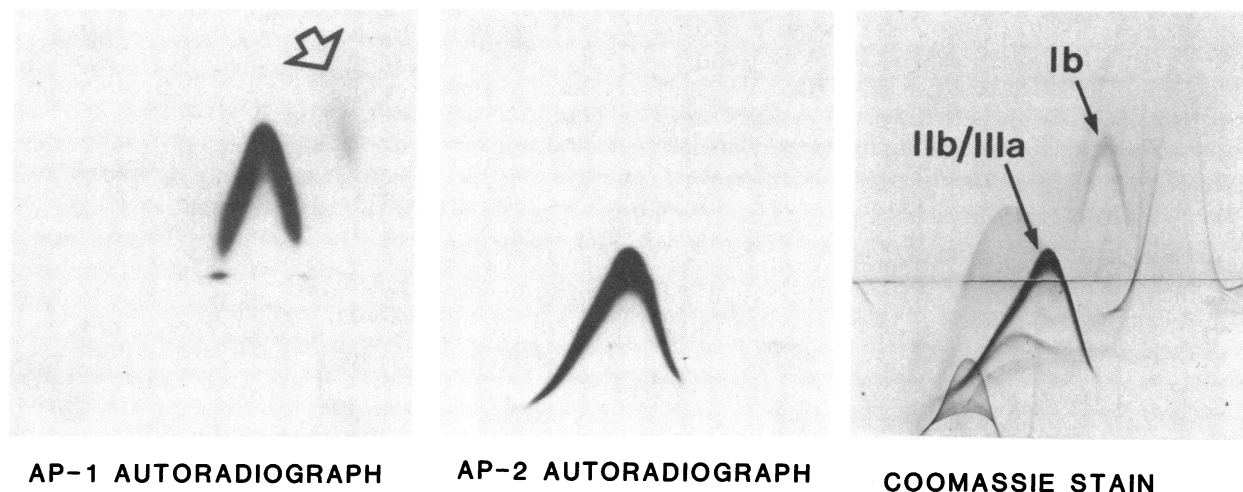


FIGURE 1 Specificity of AP-1 and AP-2 as determined by crossed immunoelectrophoresis. Crossed immunoelectrophoresis of Triton X-100 solubilized platelets was carried out against rabbit anti-human platelet antisera (unlabeled) and labeled monoclonal antibody (AP-1 or AP-2). Slides were stained and autoradiography performed, permitting the identification of the specificity of the monoclonal antibodies.

IgG). Triton X-100 extracts of human platelets were subjected to crossed immunoelectrophoresis against unlabeled rabbit antiplatelet antibody and labeled AP-1 or AP-2. The slides were stained with Coomassie Blue R and autoradiographed. The radiolabeled precipitates therefore identified the reactivity of the monoclonal antibodies as shown in Fig. 1. The AP-1 labeled the GPIb immunoprecipitate and a fainter immunoprecipitate (note arrow) that corresponds to the location of the glyocalicin immunoprecipitate. AP-2 labeled the GPIIb/IIIa immunoprecipitate and was specific for the complex. If divalent cation was chelated with EDTA and the complex of GPIIb/IIIa dissociated (13), neither the free GPIIb nor the free GPIIIa immunoprecipitate was radiolabeled.

AP-1 was a potent inhibitor of ristocetin-induced platelet agglutination with a titer of 1:3,000. In addition, AP-1 blocked >99% of the binding to platelets of radiolabeled VIIIIR:Ag as induced by ristocetin.

Monoclonal antibody binding assay on whole blood samples. Fig. 2 illustrates the results on the 23 normal individuals, 6 patients with Glanzmann's thrombasthenia, and 3 patients with Bernard-Soulier syndrome. With whole blood, the Glanzmann's patients had normal AP-1 binding, whereas the Bernard-Soulier patients had <2% binding. AP-2 binding studies demonstrated that the Glanzmann patients had <13% AP-2 binding, whereas the Bernard-Soulier patients had normal AP-2 binding. Of note are the three Glanzmann's patients who had negligible AP-2 binding, whereas there were three others that had greatly reduced AP-2 binding. None of the former group had

demonstrable GPIIb/IIIa on glycoprotein analysis, whereas the latter three had reduced but detectable GPIIb/IIIa on glycoprotein analysis.

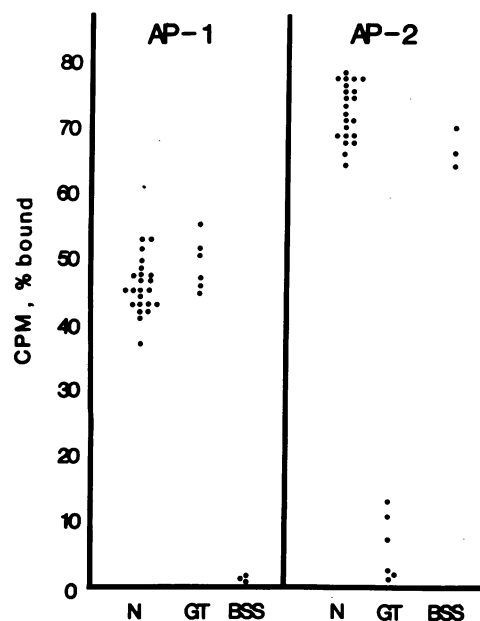


FIGURE 2 Quantitative binding of radiolabeled AP-1 and AP-2 to platelets in whole blood. A whole blood assay for AP-1 and AP-2 binding was carried out against normal individuals (N), six patients with Glanzmann's thrombasthenia (GT), and three patients with Bernard-Soulier syndrome (BSS). The percent of the radioactivity bound reflected the binding of the monoclonal antibodies to their respective glycoproteins.

Quantitative AP-1 and AP-2 monoclonal antibody binding assay on platelet-rich plasma. With platelet-rich plasma ($250,000/\text{mm}^3$), two of the Bernard-Soulier patients and one of the Glanzmann patients were compared with serial dilutions of normal platelet-rich plasma. The severe Glanzmann patient bound normal AP-1 but less AP-2 than a 1:256 dilution of normal platelet-rich plasma. Conversely, the Bernard-Soulier patients bound increased AP-2 (approximately twice normal) but less AP-1 than a 1:256 dilution of normal platelet-rich plasma.

DISCUSSION

The diagnosis of Glanzmann's thrombasthenia and Bernard-Soulier syndrome is based upon characteristic platelet function testing (1, 4, 5), crossed immunoelectrophoresis of Triton X-100 solubilized platelets (12, 13), or detailed glycoprotein analysis (1, 2, 12). In one study, GPIb was quantitated by densitometric scanning of periodic acid-Schiff-stained polyacrylamide gels of platelet glycoproteins (18). Bernard-Soulier platelets were found to have 37% of normal GPI. Although that study evaluated two of the same patients (A.J. and T.H.) as our study, our monoclonal assay measures a single antigenic locus. Because the previous study could not demonstrate any glycocalicin and our monoclonal antibody crossreacts with glycocalicin, this may explain why our assay appears to demonstrate a greater deficiency. When platelet-rich plasma from the same patient (A.J.) was compared with normal platelets with our assay, <0.4% of the normal AP-1 binding was present. Because a monoclonal antibody measures only one antigenic site, we are not able to conclude that there is a similar reduction of the entire GPIb molecule.

Although other investigators (7) have studied isolated platelets from patients with Glanzmann's thrombasthenia with a monoclonal antibody to GPIIb/IIIa, whole blood was not tested. We were able to detect the surface platelet glycoprotein deficiency with monoclonal antibodies to specific platelet glycoproteins in a whole blood assay. This is particularly helpful in diagnosing Bernard-Soulier syndrome, in which platelet separation is difficult due to the increased platelet size (1, 3). We found the assay to give consistent results even on 36-h-old refrigerated whole blood anticoagulated with acid citrate dextrose. Thus, shipping of samples is possible for this determination. Whereas, glycoprotein analysis is a moderately tedious determination, results with our method are available within 2 h of receipt of the sample. Although not studied, this technique would permit the diagnosis of these disorders in newborns, where platelet function testing

is often impossible due to sample volume. This assay, on the other hand, requires <1 ml of whole blood.

Although more precise quantitation of glycoproteins is possible if platelet-rich plasma is used, the degree of separation of normal and abnormal platelets in the more easily performed whole blood assay makes it a better screening test. This whole blood assay is capable of distinguishing between type II Glanzmann's thrombasthenic patients and severe type I thrombasthenic patients. In two instances, the Glanzmann patients had been thought to be severe until this assay was performed. Subsequent glycoprotein analysis demonstrated detectable GPIIb/IIIa, thereby substantiating their type II classification.

There is no consensus as to whether the platelets from Bernard-Soulier syndrome patients should be studied at equivalent platelet counts or equivalent platelet mass. It was of interest that although these patients were thrombocytopenic, their AP-2 binding was within the normal range on the whole blood assay. When the platelets were run at equivalent concentration to normal platelets (platelet-rich plasma), the AP-2 binding was about twice normal, suggesting either that Bernard-Soulier platelets have twice the mass of normal platelets or contain increased amounts of GPIIb/IIIa.

Preliminary results with other blood cells have failed to show AP-1 or AP-2 binding unless the preparation is contaminated with platelets. Studies on heterozygotes or carriers of these disorders may provide a method of carrier detection without the more difficult glycoprotein analysis that previously had been necessary.

ACKNOWLEDGMENTS

We wish to thank Wendy Stinebaugh for preparation of the manuscript.

This work is supported by grants 80-803 and 82-884 of the American Heart Association; Midwest Athletes against Childhood Cancer fund, Milwaukee, WI; and grant HL 13629 of the National Heart, Lung, and Blood Institute.

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