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Research Article

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ABSTRACT We used the hybridoma technique to characterize further the platelet glycoprotein abnormality in Glanzmann's thrombasthenia. Spleen cells from Balb/c mice immunized with human platelets were fused to mouse myeloma cell line Sp2/0-Ag14. Hybridoma lines producing a variety of antiplatelet antibodies were isolated by hypoxanthine-aminopterin-thymidine selection and cloned, and purified monoclonal IgG from six lines was prepared. One of these lines, 8aB5-9, produced an antibody, *Tab*, that binds to a protein on normal but not thrombasthenic platelets. We isolated this protein from Triton X-100 solubilized normal platelet membranes by affinity chromatography on *Tab*-Sepharose. As determined by SDS polyacrylamide gel electrophoresis, the isolated protein is a complex of glycoproteins IIb and IIIa, because the two subunits comigrate with glycoproteins IIb and IIIa of whole platelets and show identical changes in mobility after disulfide bond reduction. We prepared ^{125}I-*Tab* to determine the number of glycoprotein IIb-IIIa complexes on normal and thrombasthenic platelets by a direct binding assay. Platelets from 17 normal donors bound 39,000±4,600 (SD) *Tab* molecules/platelet. Platelets from four patients with thrombasthenia lacked *Tab* binding sites (<5%). Five obligate and four presumed heterozygotes for thrombasthenia bound 24,500±5,800 *Tab* molecules/platelet. The platelet alloantigen, Pl^{A1}, is not that recognized by *Tab*, because platelets from three Pl^{A1}-negative subjects bound *Tab* normally. Studies with the *Tab* antibody have (*a*) enabled quantitation of the number of glycoprotein IIb-IIIa complexes on normal platelet membranes, (*b*) demonstrated that thrombasthenic homozygotes lack and heterozygotes have a partial deficiency of this complex, and (*c*) made possible the isolation of this membrane protein which may be required for normal platelet aggregation and clot retraction.

INTRODUCTION

Glanzmann's thrombasthenia, a congenital platelet disorder with an autosomal recessive pattern of inheritance, is characterized by a normal platelet count, prolonged bleeding time, abnormal clot retraction, and absent platelet aggregation in response to ADP, thrombin, and epinephrine (1–3). Nurden and Caen (4), using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[1] presented evidence that a major periodic acid-Schiff staining platelet membrane glycoprotein was deficient in thrombasthenia (4). Phillips and Poh Agin, using two-dimensional SDS gel electrophoresis, later showed that two membrane glycoproteins, now designated as IIb and IIIa (or III), were reduced in the disease (5). Their findings were subsequently confirmed by Nurden and Caen (6) and by Jamieson et al. (7), using various modifications for separating platelet proteins by SDS-PAGE. Studies using crossed immunoelectrophoresis of Triton X-100 solubilized platelets have suggested that glycoproteins IIb and IIIa may form a discrete structure in the membrane since two proteins that comigrate with glycoproteins IIb and IIIa can be eluted from a single immunoprecipitate produced with rabbit antiplatelet antibodies or an alloantibody from a multiply transfused thrombasthenic patient (8). Because several proteins

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[1] *Abbreviations used in this paper*: CM, complete medium; PAGE, polyacrylamide gel electrophoresis; Pl^{A1}, a platelet specific alloantigen; TCM, modified complete medium.

migrate similarly on SDS gels (6), it has been difficult to quantitate the amount of glycoproteins IIb and IIIa in normal and thrombasthenic platelets. Furthermore, it has not been possible to isolate these glycoproteins.

The production of monoclonal antibodies through the technique of somatic cell hybridization developed by Köhler and Milstein (9) has proven useful in studying cell surfaces, particularly in the analysis of differentiation and tumor-specific antigens (10–15). We have applied this technique to the study of platelet surface components and have developed a number of hybridoma cell lines producing a variety of antiplatelet antibodies. We report here that one of these cloned cell lines produces a monoclonal antibody, designated *Tab*, that is directed against a platelet membrane complex consisting of glycoproteins IIb and IIIa. We have used this antibody to enumerate the number of IIb-IIIa complexes on normal platelets and to document the virtual absence of this protein on the surface of thrombasthenic platelets. We have further isolated the glycoprotein IIb-IIIa complex from Triton X-100 solubilized human platelet membranes by affinity chromatography on *Tab*-Sepharose.

METHODS

Materials. Materials were obtained from the following sources: Dulbecco's modified Eagle medium, horse, calf, and fetal calf serums from KC Biological, Inc. (Lenexa, Kan.); penicillin-streptomycin, nonessential amino acids, and Earle's basic salt solution from Gibco Laboratories (Grand Island Biological Co., Grand Island, N. Y.); carrier-free [^{125}I]sodium iodide from New England Nuclear (Boston, Mass.) and Amersham Corp. (Arlington Heights, Ill.); tissue culture flasks and centrifuge tubes from Corning Glass Works (Science Products Div., Corning, N. Y.) and Falcon Labware (Div. of Becton, Dickinson & Co., Oxnard, Calif.); cluster 24 dish wells from Costar Data Packaging (Cambridge, Mass.); the IgG fraction from goat antisera to mouse IgG from Gateway Immunosera Co. (St. Louis, Mo.); nonimmune mouse IgG from N. L. Cappel Laboratories, Inc. (Cochranville, Pa.); DEAE cellulose (DE52) from Whatman Inc. (Clifton, N. J.); Apiezon oil from James G. Biddle Co. (Plymouth Meeting, Pa.); cryoprotective medium from Microbiological Associates (Walkersville, Md.); 2,6,10,14-tetramethyl-pentadecane ("Pristane") from Aldrich Chemical Co., Inc. (Milwaukee, Wisc.). All other chemicals were reagent grade products of Sigma Chemical Co. (St. Louis, Mo.) or Fisher Scientific Co. (Pittsburgh, Pa.).

Cell lines. The mouse myeloma cell line used for fusion was Sp2/0-Ag14, a hypoxanthine guanine phosphoribosyltransferase-deficient line resistant to 8-azaguanine that synthesizes no immunoglobulin (16). Swiss mouse 3T3 cells were used as a feeder layer for cloning. Both cell lines were a gift from Dr. Brian Clevinger (Washington University).

Media. Sp2/0-Ag14 cells and hybridoma cells were maintained in complete medium (CM) consisting of Dulbecco's modified Eagle medium containing 2 mM glutamine and supplemented with 10 U/ml penicillin, 10 μg/ml streptomycin, 0.45% glucose, 0.37% sodium bicarbonate, 0.1 mM nonessential amino acids, and 15% horse serum. Sp2/0-Ag14 cells were grown periodically in CM containing 20 μg/ml 8-azaguanine to eliminate hypoxanthine-guanine-phosphoribosyl-transferase-positive revertants. Hypoxanthine-aminopterin-thymidine medium consisted of CM supplemented with 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine (17). Hypoxanthine-thymidine medium was identical to hypoxanthine-aminopterin-thymidine medium except that aminopterin was omitted. 3T3 cells were maintained in CM supplemented with 10% calf serum instead of horse serum. During cloning, cells were grown in CM supplemented with 10% horse serum and 5% calf serum. Cell fusions were performed in complete modified medium (TCM), a serum-free variation of CM consisting of Dulbecco's modified Eagle's medium supplemented with 0.35% sodium bicarbonate, 0.1% glucose, 0.03 M sodium chloride, 0.025 M Hepes, adjusted to pH 7.4 with sodium hydroxide. Agar (2%) in Earle's basic salt solution was melted and diluted 1:10 into cloning medium at 44°C for cell cloning.

Immunization. Balb/c mice were immunized intraperitoneally with 10^9 human platelets suspended in 200 μl of 0.15 M sodium chloride, 20 mM Tris-HCl, pH 7.4, containing 1 National Institutes of Health U bovine thrombin. Mice used in these experiments were boosted with a similar platelet preparation from different donors monthly for 6 mo with a final boost 3 d before cell fusion.

Fusion. Cell fusion was performed with polyethylene glycol essentially as described by Davidson and Gerald (18). Sp2/0-Ag14 cells and a cell suspension from the spleens of two immunized mice were each washed twice in TCM at 4°C. Approximately 3×10^8 spleen cells and 3×10^7 Sp2/0-Ag 14 cells were mixed in a 50-ml plastic centrifuge tube and pelleted at 250 *g* for 7 min. The supernate was removed and the cell pellet loosened by gentle tapping of the bottom of the tube. All subsequent steps were performed at 37°C. 1 ml of a 50% solution of polyethylene glycol 1500 in TCM was added dropwise over a 1-min period with gentle swirling. During the next minute, 1 ml of TCM was added, followed over the next 4 min by the dropwise addition of 20 ml of TCM. The cells were pelleted again and resuspended in 48 ml of CM. 2 ml of the suspension were incubated in each well of a cluster 24 dish in a humidified atmosphere containing 5% CO_2 at 37°C. Half of the medium was replaced with hypoxanthine-aminopterin-thymidine medium daily for the next 3 d. By 2 wk of incubation, growth of hybrid cells was seen in all the wells and over the next 2–3 wk the hypoxanthine-aminopterin-thymidine medium was gradually replaced by hypoxanthine-thymidine (HT) medium, then by CM. Hybridoma cells from wells containing antiplatelet antibody (see screening procedure below) were cloned by limiting dilution in 0.2% agar in cluster dish wells that contained a feeder layer of 3T3 cells. After 2 wk of growth individual clones were removed from the soft agar, using a Pasteur pipette and dissecting microscope, and transferred to individual cluster dish wells containing CM where they were grown further and rescreened for antiplatelet antibody production. Cells from positive clones were suspended in cryoprotective medium supplemented with 20% fetal calf serum and frozen in liquid nitrogen.

Purification of monoclonal IgG. The protein concentration of mouse IgG was estimated from absorbance at 280 nm using an estimated $E^{1\%}_{280}$ of 14.3 (19).

Approximately 10^7 cloned antiplatelet antibody producing hybridoma cells were injected intraperitoneally into Balb/c mice previously "primed" with a 0.5 ml i.p. injection of Pristane. After 1–2 wk ascitic fluid containing concentrated antibody was collected, allowed to clot, and clarified by centrifugation. Mouse IgG was precipitated with 50% ammonium sulfate, dissolved in 0.01 M potassium phosphate, pH 7.4, and dialyzed at 4°C overnight against the same buffer. The dialyzed material was applied to a column of DEAE-cellulose (7–10 ml bed volume per ml of ascites) equilibrated

in the 0.01 M potassium phosphate buffer. The column was eluted using a 0–0.5-M sodium chloride gradient in the phosphate buffer. Mouse IgG eluted at 0.02 M sodium chloride, with a yield of 1–2 mg of IgG per ml of ascites.

Protein iodination. IgG was labeled with [^{125}I]iodide by a modified chloramine T procedure (20). Protein was separated from free iodide on a Sephadex G-25 column. For antiplatelet antibody screening assays, 5 μg of goat antimouse IgG was labeled to high specific activity (40–80 μCi/μg protein) and stored at −20°C in 0.15 M sodium chloride, 0.02 M Tris HCl, pH 7.4, and 5 mg/ml bovine serum albumin. For direct binding studies, 1 mg of *Tab* IgG was labeled to a specific activity of 50–100 cpm/ng and stored at −20°C at a concentration of 200 μg/ml in the Tris saline buffer without the addition of albumin.

Binding assays. All reactions were at room temperature. In the indirect binding assay used to screen for antiplatelet antibody, 10^8 platelets in 1 ml of 0.15 M sodium chloride, 0.02 M Tris HCl, pH 7.4, 1 mg/ml glucose, 5 mg/ml bovine serum albumin, 10 mM EDTA, and 50 μg/ml nonimmune human IgG were incubated for 15 min in Brinkmann 1.5-ml conical plastic centrifuge tubes (Brinkmann Instruments, Inc., Westbury, N. Y.). Then 50 μl of culture medium from hybridoma wells or from cloned hybridoma cell lines were added. In control tubes, 50 μl of culture medium not incubated with cells or medium incubated with nonsecreting Sp2/0-Ag14 cells were added. After 30 min the platelets were collected by centrifugation at 2,000 *g* for 10 min, washed once, centrifuged again, and resuspended in 0.5 ml of the same buffer. ^{125}I-labeled goat anti-mouse IgG, 400,000 cpm, was then added. After 30 min the reaction mixture was layered over 0.5 ml of a 9:1 mixture of *n*-butyl phthalate (specific gravity 1.046) and Apiezon A oil (specific gravity, 0.865). Platelets were collected by centrifugation at 12,000 *g* in a Brinkmann microfuge for 1.5 min. After the supernate and oil were aspirated, the tip of the tube containing the platelet pellet was cut off with a scalpel and the radioactivity measured.

Direct binding of monoclonal antibody to platelets was performed similarly to that reported previously for Factor X_a binding (21). Reaction mixtures in 12 × 75-mm polystyrene tubes contained 10^8 platelets/ml in the same buffer as above minus EDTA. After preincubation with 50 μg/ml of nonimmune human IgG for 15 min, varying amounts of ^{125}I-*Tab* were added. After 30 min, 0.4 ml (4×10^7 platelets) of the reaction mixture were layered over 0.5 ml of the *n*-butyl phthalate-Apiezon A oil mixture as in the indirect binding assay and centrifuged at 12,000 *g* for 1.5 min. To determine the concentration of free ^{125}I-*Tab*, 0.2 ml of the supernate was sampled for radioactivity. The remaining supernate and oil were aspirated. The tip of the tube containing the platelet pellet and bound ^{125}I-*Tab* was then cut off and the radioactivity determined.

Affinity chromatography. Nonimmune mouse IgG or monoclonal *Tab* IgG were coupled to cyanogen bromide-activated Sepharose 4B (1.5–2 mg protein/ml of Sepharose) (22). In a typical experiment, 50×10^9 washed platelets were resuspended in 0.15 M sodium chloride, 0.02 M Tris HCl, pH 7.4, containing 2.5 mM EDTA, and 6 mM diisopropylfluorophosphate. The platelets were sonicated (three times at 100 W for 20 s at 4°C) and centrifuged at 100,000 *g* for 30 min at 4°C in an ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The crude membrane fraction was resuspended at 37°C for 30 min in 8 ml of 0.3 mM sodium phosphate, pH 7.4, containing 2.5 mM EDTA to reduce the amount of actin contamination (23, 24). The membranes were recentrifuged and suspended in 10 ml of 0.15 M sodium chloride, 0.02 M Tris HCl, pH 7.4, 2.5 mm EDTA, 6 mM diisopropylfluorophosphate, 1% Triton X-100, and the preparation was centrifuged again at 100,000 *g* for 30 min. The supernate was first applied to a 10-ml column of uncoupled Sepharose 4B equilibrated in 0.15 M sodium chloride, 0.02 M Tris HCl, pH 7.4, 1% Triton X-100. The flow-through fraction from this column, which removes material adsorbing nonspecifically to Sepharose, was then applied to a 1–3-ml *Tab*-Sepharose 4B column previously eluted with 0.05 M diethylamine, pH 11.5, 1% Triton X-100, and reequilibrated in 0.15 M sodium chloride, 0.02 M Tris HCl, pH 7.4, 1% Triton X-100. The column was then washed with 1 M sodium chloride, 1% Triton X-100; then 0.1 M Tris HCl, pH 7.4, 1% Triton X-100; and then 0.15 M sodium chloride, 0.02 M Tris HCl, pH 7.4, 1% Triton X-100. Bound platelet protein was then eluted with 0.05 M diethylamine, pH 11.5, 1% Triton X-100 as described by Parham (25). The eluate was neutralized with 2 M Tris HCl, pH 7.4, 1% Triton X-100, dialyzed against 0.15 M sodium chloride, 0.02 M Tris HCl, pH 7.4, 6 mM diisopropylfluorophosphate, 2.5 mM EDTA, and stored at 4°C.

Other methods. Washed platelets were isolated from blood anticoagulated with EDTA as previously described (26). Human lymphocytes were prepared from fresh defibrinated blood by centrifugation over Ficoll-Hypaque by the method of Bøyum (27) and washed three times in 0.15 M sodium chloride, 0.02 M Tris-HCl, pH 7.4, before use. Human erythrocytes from EDTA-anticoagulated blood were collected by centrifugation at 120 *g* for 10 min and washed three times in 0.15 M sodium chloride before use. SDS-PAGE was performed according to Laemmli (28). Samples to be electrophoresed were heated at 100°C in SDS sample buffer for 5 min. Reduced samples contained 5% 2-mercaptoethanol. Radioactive iodide was measured in a Beckman Gamma 300 γ counter (Beckman Instruments, Inc., Fullerton, Calif.).

Platelets from Pl^{A1}-negative subjects and from thrombasthenic patients and family members. Blood from three Pl^{A1}-negative subjects was obtained through the Missouri-Illinois Red Cross Regional Blood Services.

Four patients with thrombasthenia were studied: R.F. (St. Louis), S.L. and P.C. (a sister and brother from California), and H.M. (Chicago). All the patients had normal platelet counts, markedly prolonged bleeding time, decreased or absent clot retraction, and lack of platelet aggregation in response to ADP or epinephrine. One patient (H.M.) has been reported previously (29). All of the patients had a history of recurrent mucocutaneous bleeding, although P.C. had less severe bleeding symptoms. Seven family members of S.L. and P.C. and four family members of R.F. were also studied. Direct *Tab* binding assays were performed on freshly prepared platelets from R.F. and his family members. It was subsequently determined that the number of *Tab* binding sites on platelets was not affected if blood samples collected in 5.8 mM EDTA in plastic syringes were left at room temperature for up to 24 h. Therefore, blood samples from the remaining patients and family members were flown to St. Louis and the platelets studied within 24 h of phlebotomy. Concurrent control blood samples were drawn and assayed in similar fashion.

RESULTS

Preparation of Tab. In one of eight cell fusion experiments, screening with the indirect binding assay showed that cells from all 24 cluster dish wells produced antiplatelet antibodies. Cells from each well were frozen in liquid nitrogen. Cells from six wells, arbitrarily chosen, were cloned and monoclonal IgG was purified from ascites prepared from six cloned lines. Indirect immunofluorescence studies in which thin sections from a variety of human tissues were

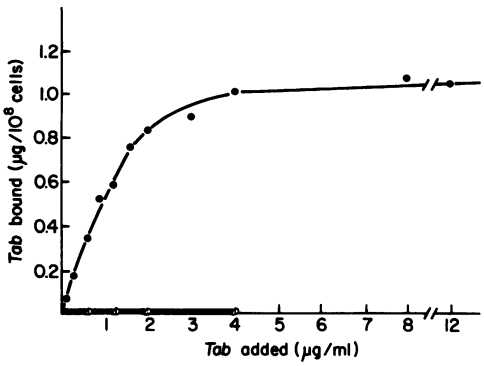

FIGURE 1 Steady-state binding of ^{125}I-*Tab* to human platelets (●), erythrocytes (○), and lymphocytes (△). Reaction mixtures initially contained 10^8 cells/ml (preincubated with 50 μg/ml nonimmune human IgG) and varying amounts of ^{125}I-*Tab* in 0.15 M sodium chloride, 0.02 M Tris-HCl, pH 7.4, 5 mg/ml bovine serum albumin, and 1 mg/ml glucose.

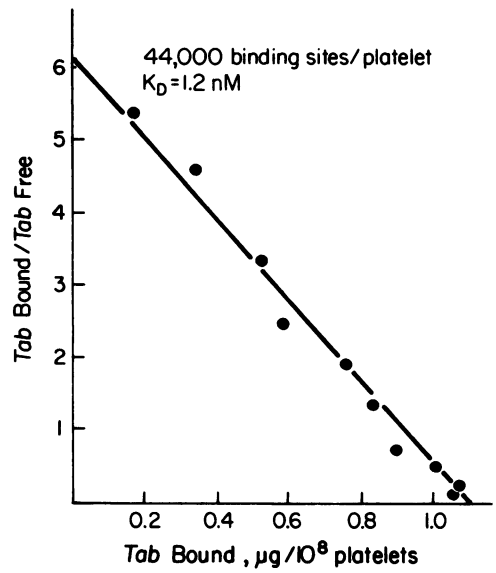

FIGURE 2 Scatchard plot of the ^{125}I-*Tab* binding to platelets from Fig. 1.

reacted first with mouse IgG, then with fluorescein-labeled goat antibody to mouse IgG, showed that five of the six monoclonal antibodies bound to one or more cell types in addition to platelets.[2] The remaining antibody, derived from clone 8aB5-9, bound only to platelets and was studied in detail. This antibody, designated *Tab*, was shown to be an IgG by Ouchterlony immunodiffusion using goat antibody to mouse IgG and was purified to homogeneity as determined by SDS-PAGE (data not shown).

Affinity of Tab for platelets. We performed direct binding studies with ^{125}I-*Tab* to determine the number of *Tab* binding sites on human platelets. In preliminary experiments we found that *Tab* binding is 50% complete after 5 min and is at least 95% complete by 30 min of incubation (data not shown). Therefore all binding studies were performed with a 30-min incubation with ^{125}I-*Tab*. A typical equilibrium binding experiment is shown in Fig. 1. Binding data are uncorrected for nonspecific binding since a 100-fold excess of unlabeled *Tab* added to reaction mixtures displaced 99.8% or more of bound ^{125}I-*Tab*. The 0.2% undisplaced radioactivity corresponds to values of trapped water in the platelet pellets determined previously using ^{131}I-ovalbumin (30). ^{125}I-*Tab* did not bind to erythrocytes or to lymphocytes (Fig. 1). A Scatchard plot of the ^{125}I-*Tab* binding data is shown in Fig. 2. We found a single class of binding sites to which *Tab* binds with high affinity. In this experiment the dissociation constant for ^{125}I-*Tab* was 1.2 nM with 44,000 binding sites/platelet (1.1 μg/10^8 platelets). The ^{125}I-*Tab* from ascites is nearly homogeneous since 90% of added ^{125}I-*Tab* binds to platelets when 50 ng of *Tab* is added to 10^8 platelets. Addition of *Tab* to platelets did not cause release of [^{14}C]serotonin and binding of ^{125}I-*Tab* was identical in unstimulated or thrombin-treated platelets (data not shown).

Binding of Tab to thrombasthenic platelets. In initial double antibody immunoprecipitation studies, we discovered that *Tab* precipitated two polypeptides of molecular weights 130,000 and 115,000 as determined by SDS-PAGE from Triton X-100 solubilized human platelets (data not shown). This suggested that *Tab* might be directed against a membrane complex consisting of glycoproteins IIb and IIIa, which have molecular weights of ~130,000 and 115,000 and which are deficient in thrombasthenia (5). To examine this possibility we measured ^{125}I-*Tab* binding to platelets from patients with thrombasthenia. Fig. 3 shows the binding of ^{125}I-*Tab* to platelets from a normal subject, a patient with thrombasthenia (S.L.), and the patient's

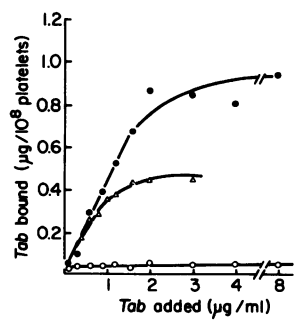

FIGURE 3 Steady-state binding of ^{125}I-*Tab* to normal and thrombasthenic platelets. Reaction mixtures were as in Fig. 1. ●, Normal; △, thrombasthenic heterozygote; ○, thrombasthenic homozygote.

[2] McEver, R. P., C. E. Bell, and P. W. Majerus. Unpublished observations.

father, an obligate heterozygote. The platelets from the patient bound virtually no *Tab* (<5% of normal). Platelets from the patient's father had approximately half the number of *Tab* binding sites as the control subject.

Fig. 4 summarizes ^{125}I-*Tab* binding to platelets of normal subjects, patients with thrombasthenia, and members of two families of thrombasthenic patients. Platelets from 17 normal subjects bound 39,400±4,600 (SD) *Tab* molecules per platelet with a dissociation constant (K_D) of 0.73±0.20 (SD) nM. Four patients with thrombasthenia from three families bound <2,000 *Tab* molecules (<5% of normal) per platelet. Five obligate heterozygotes for thrombasthenia were studied: a son and the parents of S.L. and the parents of R.F. Four of these family members showed intermediate levels of *Tab* binding sites on their platelets and were clearly below the normal range (defined as the mean±2 SD of the 17 normal subjects). Normal levels of *Tab* molecules were bound to the platelets of one obligate heterozygote, the father of R.F., on two separate occasions. This may indicate some variation in the amount of glycoprotein in thrombasthenic heterozygotes, although lack of true paternity was not rigorously excluded in this case. Six other family members who were potential heterozygotes for thrombasthenia were studied. Two of these bound <20,000 *Tab* molecules per platelet and were clearly abnormal. Two others bound ~29,000 molecules per platelet and may also be heterozygotes. Two others appeared to be normal since their platelet *Tab* binding sites were in the upper part of the normal range. The mean number of *Tab* binding sites for the five obligate heterozygotes and the four other family members whose platelets had decreased

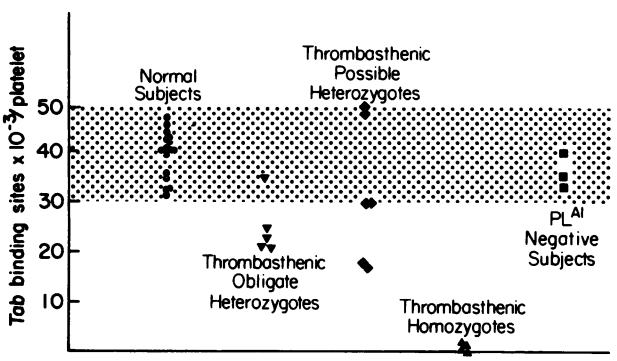

FIGURE 4 ^{125}I-*Tab* binding sites per platelet from normal subjects, thrombasthenic patients and family members, and Pl^{A1}-negative subjects. The shaded normal range (▨) was defined as the mean (39,400) ±2 SD (9,200) of the 17 normal subjects. The obligate heterozygotes for thrombasthenia were four parents and one son of the patients. The possible heterozygotes were siblings or nephews of the patients. The maximum number of binding sites for each subject was obtained using a double reciprocal plot of the least mean squares best fit for the ^{125}I-*Tab* binding data.

Tab binding was 24,500±5,800 (SD) per platelet. The mean K_D was 0.51±0.20 nM, similar to normal subjects and suggesting that the decrease in binding was due to a decrease in binding sites rather than an altered affinity.

Platelets from the 2% of the normal population lacking the platelet alloantigen, Pl^{A1}, have normal levels of glycoproteins IIb and IIIa as measured by SDS-PAGE (31). The Pl^{A1} antigen is also reduced in platelets from patients with thrombasthenia (31) and it has been suggested that the antigen resides on glycoprotein IIIa (32). We therefore performed *Tab* binding assays on the platelets of three Pl^{A1}-negative donors. As shown in Fig. 4 the platelets of all three donors bound *Tab* normally, indicating that *Tab* does not recognize the Pl^{A1} antigen.

Isolation of glycoprotein IIb-IIIa. The *Tab* binding experiments indicate that *Tab* binds to a major surface structure of platelets that is lacking in platelets from thrombasthenic patients and reduced to an intermediate degree in thrombasthenic heterozygotes. To confirm that this structure consists of a complex of glycoproteins IIb and IIIa, we isolated the complex from Triton X-100 solubilized platelet membranes on a *Tab*-Sepharose affinity column as described in Methods. Fig. 5 shows a Coomassie blue-stained SDS polyacrylamide gel of the bound protein eluted from the *Tab*-Sepharose column. The unreduced platelet protein consists of two polypeptides of apparent molecular weights of 142,000 and 90,000. There is also a small

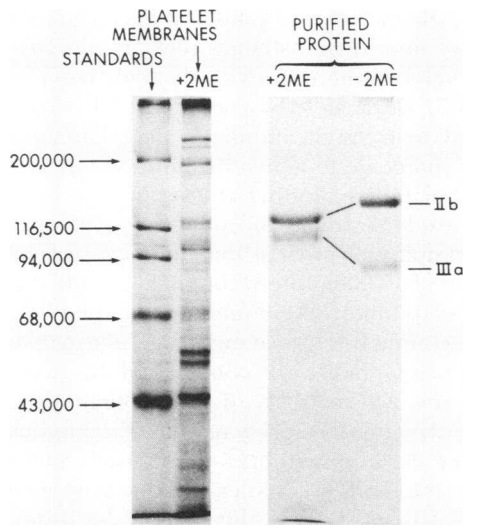

FIGURE 5 SDS gel electrophoresis on a Coomassie blue-stained 7.5% polyacrylamide slab gel of platelet glycoprotein IIb-IIIa isolated by affinity chromatography on *Tab*-Sepharose. Lanes left to right: lane 1, molecular weight standards; lane 2, Triton X-100 solubilized platelet membranes before application to the affinity column, reduced; lane 3, glycoprotein IIb-IIIa eluate from *Tab*-Sepharose, reduced; lane 4, glycoprotein IIb-IIIa eluate, unreduced. 2 ME, 2 mercaptoethanol.

amount of aggregated protein that just enters the 7.5% acrylamide gel. In the reduced platelet protein, the apparent molecular weights of the two polypeptides have been altered to 127,000 and 115,000. There is also a faint band of protein with molecular weight 43,000, which may represent actin and a faintly staining band near the tracking dye representing a small peptide released by reduction of the 142,000-mol wt glycoprotein IIb (33). The aggregated protein is not seen in the reduced gel, suggesting that it may consist of the two major polypeptides linked by disulfide bonds in larger complexes to actin. The apparent molecular weights of the two polypeptides and their characteristic changes in migration after reduction are similar to those reported previously for glycoproteins IIb and IIIa (6, 33). Both of these proteins also stain with periodic acid-Schiff and comigrate with periodic acid-Schiff-stained glycoproteins IIb and IIIa of whole platelets (data not shown). In control experiments, neither polypeptide bound to a Sepharose 4B column to which nonimmune mouse IgG had been coupled. These data indicate that the protein complex isolated from human platelets with *Tab* consists of glycoproteins IIb and IIIa.

DISCUSSION

In this study, we have demonstrated that the 8aB5-9 cloned hybridoma cell line produces a monoclonal antibody, *Tab*, directed against a major platelet surface structure deficient in platelets of patients with thrombasthenia and reduced to an intermediate degree in thrombasthenic heterozygotes. We have also isolated this complex and demonstrated that it consists of two platelet glycoproteins previously designated as IIb and IIIa (5–7). Because *Tab* is monoclonal, it is presumably directed to a single antigenic determinant on each surface molecule to which the antibody binds. Hence, the radiolabeled binding studies we have described can be used to estimate the number of glycoprotein IIb-IIIa complexes present on normal platelets. The estimate of 40,000 protein complexes could differ from the true number. For example, the platelet surface proteins might be close enough together so that a single divalent IgG molecule could bind to two IIb-IIIa complexes. Alternatively, if the polypeptides IIb and IIIa are structurally closely related, *Tab* might bind to a common determinant present on each polypeptide. Finally, the IIb-IIIa complex could consist of more than a single IIb and IIIa. In this case, if, for instance, *Tab* binds only to IIb and there are three IIb subunits for each IIIa in each membrane complex, then as many as three *Tab* molecules might bind to the same complex. We attempted to localize the antigenic determinant by overlaying ^{125}I-labeled *Tab* on glycoproteins IIb and IIIa separated by SDS-PAGE, then performing autoradiography. However, no ^{125}I-*Tab* binding was seen, presumably because the antigen was denatured by SDS and was no longer recognized by the antibody. We favor the hypothesis that *Tab* binds only to IIb or to IIIa, and that the polypeptide to which *Tab* does not bind is also isolated on *Tab*-Sepharose because the two polypeptides are subunits of the same membrane complex and remain associated during Triton X-100 solubilization. It is also possible that *Tab* binds to a determinant composed of parts of both IIb and IIIa. Further studies are required to determine whether the complex consists of one IIb and one IIIa subunit or some other mixture of subunits. If the complex is a 1:1 mixture, 40,000 is an accurate estimate of the number of complexes per platelet.

Exogenous fibrinogen is required for the aggregation of washed platelets by ADP and epinephrine (34–36). Several investigators have recently shown that ^{125}I-labeled fibrinogen binds in a saturable and initially reversible manner to platelets stimulated with ADP, epinephrine, and thrombin (37–43). Three different groups found a single class of binding sites and estimated there to be ~40,000 binding sites per platelet (38–40). Two other groups found fewer receptors and suggested that there are two classes of binding sites (42, 43). The reason for the discrepancy in these estimates is not clear, but the estimate of 40,000 fibrinogen receptors (38–40) is identical to our value of 40,000 glycoprotein IIb-IIIa complexes per platelet. Thrombasthenic platelets lack the ability to bind fibrinogen (37, 42–45). These studies and our findings suggest that the glycoprotein IIb-IIIa complex is the fibrinogen binding site and is required for normal platelet aggregation. Presumably when platelets are stimulated, the glycoprotein IIb-IIIa complex undergoes a change that allows fibrinogen to bind. Bound fibrinogen molecules may then interact with each other or perhaps with a IIb-IIIa protein on another platelet to form the platelet aggregate. We presume that *Tab* recognizes a single determinant on the glycoprotein IIb-IIIa complex because of its monoclonal nature. This may explain our finding that *Tab* does not precipitate glycoproteins IIb-IIIa from a Triton extract of platelets unless a second antibody (goat anti-mouse IgG) is added in either Ouchterlony diffusion or immunoelectrophoresis experiments. That the antigen is monovalent may also explain our finding that *Tab* does not inhibit either ADP or thrombin-induced aggregation of normal platelets (data not shown). Thus a single *Tab* antibody molecule apparently does not provide sufficient steric hindrance to block fibrinogen binding and platelet aggregation.

Degos et al. (46), studied thrombasthenia using an alloantibody designated IgG-L derived from the serum of a thrombasthenic patient who had received numerous platelet transfusions. The antibody reacted in a complement fixation assay with normal platelets but not with platelets from eight other thrombasthenic patients. Platelets from seven heterozygotes for

thrombasthenia bound intermediate levels of complement. In this initial study indirect immunoprecipitation of Nonidet P-40 solubilized platelets previously labeled with ^{125}I showed only a single radioactive peak of 120,000 mol wt as determined by SDS-PAGE. In a later investigation Hagen et al. (8) performed crossed immunoelectrophoresis of human platelets using rabbit antiplatelet antibodies (8). They noted that a prominent immunoprecipitate was absent from the platelets of two thrombasthenic patients and reduced to 13% of the normal area in a third patient. They further demonstrated that the IgG-L antibody also precipitated this protein which when eluted contained two bands comigrating with glycoproteins IIb and IIIa on SDS-PAGE. This study and our results suggest that glycoproteins IIb and IIIa are associated in Triton X-100 platelet extracts and therefore probably in the platelet membrane.

Glycoproteins IIb and IIIa are thought to be integral transmembrane proteins (47) and could conceivably interact with intracellular proteins. Phillips has presented evidence that glycoproteins IIb and IIIa (III in his terminology) interact with the contractile proteins of the platelet after thrombin-induced aggregation (48). When washed, unstimulated platelets were solubilized in Triton X-100, the insoluble residue analyzed by SDS-PAGE contained primarily actin and a 255,000-mol wt polypeptide thought to be actin-binding protein. The insoluble residue from thrombin-aggregated platelets also contained myosin and glycoproteins IIb and IIIa, suggesting that the IIb-IIIa complex can interact with contractile proteins. Our finding of traces of a protein that may be actin associated with aggregates of affinity-purified glycoprotein IIb-IIIa complexes is consistent with such an interaction. It has also been suggested that glycoprotein IIIa may be related to α-actinin, a protein found in muscle and nonmuscle cells that can bind actin (49, 50).

The normal binding of ^{125}I-*Tab* to Pl^{A1}-negative platelets does not exclude the presence of the Pl^{A1} antigen on glycoprotein IIb or IIIa (32), but does indicate that *Tab* recognizes an antigen on the IIb-IIIa complex different from Pl^{A1}. Our finding that Pl^{A1}-negative platelets have normal numbers of glycoprotein IIb-IIIa complexes as measured by the ^{125}I-*Tab* binding assay is consistent with the normal staining of glycoproteins IIb and IIIa from these platelets on SDS-PAGE (31). Because individuals with Pl^{A1}-negative platelets are not reported to have abnormal bleeding symptoms, it would appear that this antigen is not required for the function of the glycoprotein IIb-IIIa complex.

The current evidence suggests a model in which the 40,000 glycoprotein IIb-IIIa complexes spanning the platelet membrane play a crucial role in platelet aggregation and clot retraction. When stimulated by thrombin or ADP, each complex undergoes a change that allows the binding of fibrinogen on the cell exterior and the interaction with proteins of the contractile system on the platelet interior. The former would allow platelet aggregation, the latter clot retraction. The isolation of the glycoprotein IIb-IIIa complex with the monoclonal antibody *Tab* should be helpful in elucidating the structure and function of this platelet membrane protein.

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