Exposure of Platelet Fibrinogen Receptors by ADP and Epinephrine

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ABSTRACT The role of fibrinogen as a cofactor for platelet aggregation was examined by measuring the binding of ^{125}I-labeled human fibrinogen to gel-filtered human platelets both before and after platelet stimulation by ADP and epinephrine. Platelet stimulation by ADP resulted in the rapid, reversible binding of fibrinogen to receptors on the platelet surface. Fibrinogen binding increased as the concentration of ADP was increased from 0.1 to 2 μM, reaching a plateau at higher ADP concentrations. Binding occurred only after platelet stimulation and in the presence of divalent cations. However, fibrinogen binding did not occur to ADP-stimulated platelets from three patients with Glanzmann's thrombasthenia. Analysis of fibrinogen binding as a function of increasing fibrinogen concentration demonstrated that maximal platelet stimulation exposed ≅45,000 binding sites per platelet with a dissociation constant of 80–170 nM. These fibrinogen binding parameters were essentially the same whether ADP or epinephrine was the platelet-stimulating agent. Thus, these studies demonstrate that platelet stimulation by ADP and epinephrine exposes a limited number of fibrinogen receptors on the platelet surface. Furthermore, these data suggest that the fibrinogen molecules bound to the platelet as a consequence of platelet stimulation are directly involved in the platelet aggregation response.

INTRODUCTION

Fibrinogen is required for normal platelet function in vivo and in vitro (1). How this protein participates in normal platelet function is as yet unclear. Although fibrinogen is stored in the platelet alpha granules (2) and may be adsorbed to the platelet surface (3), it appears that intact, extracellular fibrinogen must be present for platelet aggregation to occur. For example, fibrinogen must be added to suspensions of washed or gel-filtered platelets to restore platelet aggregation induced by ADP and epinephrine (4, 5). Similarly, thrombin-induced platelet aggregation is inhibited by the presence of anti-fibrinogen antibody (3) or by the presence of plasmin (6).

Platelet aggregation is the process by which platelets adhere to one another to form a hemostatic platelet plug (7). Because aggregation involves the surface of adjacent platelets, it is likely that fibrinogen participates in this process by interacting with the platelet surface membrane (4). In this study we have examined the role of fibrinogen in platelet aggregation by measuring the binding of radiolabeled human fibrinogen to the surface of gel-filtered human platelets both before and after platelet stimulation by ADP and epinephrine. We have demonstrated that platelet stimulation exposes a limited number of fibrinogen receptors on the surface of normal platelets. In addition, we have shown that platelets from patients with Glanzmann's thrombasthenia lack demonstrable fibrinogen receptors, which suggests that the inability of these platelets to bind fibrinogen may contribute to the abnormalities of platelet function that characterize this disease.

METHODS

Materials. Lyophilized human fibrinogen was purchased from Kabi AB, Kabi Blood Products Div., Stockholm, Sweden. Sepharose 2B, 4B, and Sephadex G-50 were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. The Sigma Chemical Co., St. Louis, Mo. supplied the ADP, epinephrine bitartrate, human albumin-fraction V, and sodium dodecyl sulfate. Acrylamide and N,N^1-methylenebisacrylamide were obtained from the Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. Coomassie brilliant blue was purchased from Bio-Rad Laboratories, Richmond, Calif. Molecular weight markers for sodium dodecyl sulfate (SDS)[1] polyacrylamide gel electrophoresis were purchased from

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[1] *Abbreviations used in this paper:* M_r, apparent molecular weight; SDS, sodium dodecyl sulfate.

BDH Chemicals Ltd., Poole, England. William F. Nye, Inc., Fairhaven, Mass. supplied Methyl silicone oil (DC200) and Hi-Phenyl silicone oil (DC550). Carrier-free ^{125}I-sodium iodide, [^{14}C]carboxylinulin, [^{14}C]5-hydroxytryptamine binoxalate, Econofluor, and Protosol were obtained from New England Nuclear, Boston, Mass. Amersham Corp., Arlington Heights, Ill. supplied ACS scintillation fluid. Lyophilized ristocetin was purchased from Pacific Hemostasis Laboratories, Inc., Bakersfield, Calif. Antisera to factor VIII-associated protein was purchased from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. Human thrombin, 50 NIH U/ml, was purchased from Ortho Pharmaceutical Corp., Raritan, N. J. Blood from patients with Glanzmann's thrombasthenia was kindly made available to us by Dr. Margaret Johnson, Wilmington Medical Center, Wilmington, Del. All other chemicals used were of reagent grade.

Preparation of radiolabeled human fibrinogen. A 0.3% solution of Kabi fibrinogen in 0.15 M NaCl was fractionated by the precipitation of a low-solubility fibrinogen fraction with 16% saturated ammonium sulfate as described by Lipinska et al. (8). The remaining fibrinogen of higher solubility was discarded. The low-solubility fibrinogen was gel filtered over Sepharose 4B to remove contaminating von Willebrand factor and was lyophilized for storage. The protein content of the final lyophilized-fibrinogen preparation was determined by the method of Lowry et al. (9) using bovine serum albumin as the protein standard. Its thrombin clottability was >96%.

The structural integrity of the purified fibrinogen was assessed by polyacrylamide disc gel electrophoresis in SDS as described by Weber and Osborn (10). Fibrinogen was dissolved in 0.01 M sodium phosphate buffer, pH 7.2, which contained 1% SDS by heating at 100°C for 3 min. For reduced gels, 0.2 M dithiothreitol was added. Proteins were visualized with 0.1% Coomassie brilliant blue. Apparent molecular weights (M_r) were determined from the mobility of standard proteins run on concurrent gels: myosin heavy chain (M_r = 200,000), β-galactosidase (M_r = 130,000), bovine serum albumin (M_r = 68,000), ovalbumin (M_r = 43,000), and myoglobin (M_r = 17,200) or BDH molecular weight markers (BDH Chemicals Ltd., Poole, England).

The thrombin clotting time of the fibrinogen at 3 mg/ml in 0.15 M NaCl, 0.05 M Tris buffer, pH 7.8, was measured in a Fibrometer (BBL Microbiology Systems, Becton Dickinson & Co., Cockeysville, Md.) using 2.5 NIH U thrombin. Contamination of the final fibrinogen preparation by von Willebrand factor was assessed by the ability of a 10 mg/ml fibrinogen solution to agglutinate formalin-fixed platelets in the presence of ristocetin (11). In addition, the presence of von Willebrand factor antigen was determined by immunoelectrophoresis using the method of Zimmerman et al. (12). The presence of contaminating factor XIII was examined by observing the solubility of a thrombin-induced clot in 2% acetic acid (13). The ability of the fibrinogen preparation to act as a cofactor for platelet aggregation was assessed by the ability of suspensions of gel-filtered human platelets containing 1 mM Ca^{+2} to aggregate in response to micromolar concentrations of ADP and epinephrine after the addition of fibrinogen at 0.2 mg/ml.

The purified fibrinogen was radiolabeled with ^{125}I by the iodine-monochloride technique (14). Free and protein-bound ^{125}I were separated by gel filtration of the labeled fibrinogen over Sephadex G50, followed by the dialysis of the eluted protein against 0.15 M NaCl. The radioactivity of the labeled fibrinogen was measured in a Nuclear-Chicago gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.) with 85% efficiency. In general, each radiolabeled fibrinogen preparation was used for only a 2-wk period.

Preparation of gel-filtered platelets. Blood was obtained by venipuncture from drug-free, normal volunteers and was anticoagulated with 0.1 vol of 0.13 M sodium citrate. Platelet-rich plasma was prepared by centrifugation of the blood at 180 *g* for 10 min at 25°C. 3 ml of the platelet-rich plasma was gel-filtered over 30 ml Sepharose 2B in 50-ml plastic syringes (Pharmaseal Laboratories, Glendale, Calif.) (15). The platelets were eluted with Tyrode's buffer (0.14 M NaCl, 0.0027 M KCl, 0.012 M $NaHCO_3$, 0.0004 M $NaHPO_4$, pH 7.4) that contained 3.5 mg/ml human albumin and 5.5 mM glucose. The gel-filtration fractions that contained the highest apparent concentrations of platelets were pooled, and the platelet count was measured with a Coulter model Z_B particle counter (Coulter Electronics, Inc., Hialeah, Fla.). When prepared in this manner, platelets would not aggregate in response to ADP or epinephrine until Ca^{+2} and fibrinogen were added. Contamination of the gel-filtered platelets with plasma fibringen was assessed by adding labeled fibrinogen to the platelet-rich plasma before gel filtration and counting the fractions for both ^{125}I-fibrinogen and platelets. Contamination of the platelet fractions with labeled fibrinogen amounted to 0.05% of the labeled fibrinogen added to the plasma. Because the responsiveness of gel-filtered platelets to ADP and epinephrine declines after several hours, experiments with these platelets were completed within 1 h of gel filtration. The pH of the platelet suspensions remained constant during this interval.

Fibrinogen binding to gel-filtered platelets. Samples of gel-filtered platelets were mixed with labeled fibrinogen and either $CaCl_2$ or $MgCl_2$ and were allowed to equilibrate at 37°C in a water bath. ADP or epinephrine was then added, and after an initial agitation to insure mixing, incubation of the final 0.5-ml suspensions was continued at 37°C without stirring. Stirring was omitted to prevent platelet aggregation and the possible release of platelet granule fibrinogen. Absence of platelet aggregation and secretion during the mixing and incubation procedure was monitored by phase microscopy and [^{14}C]serotonin secretion. Platelet [^{14}C]-serotonin secretion was measured by the method of Jerushalmy and Zucker (16) with ACS scintillation fluid (Amersham Corp., Arlington Heights, Ill.) and a Searle Delta 300 liquid scintillation counter (Searle Diagnostics Inc., G. D. Searle & Co., Des Plaines, Ill.). The majority of the stimulated platelets appeared as individual spheres covered by numerous filipodia, although occasional aggregates of two or three platelets formed during the incubation. Secretion of [^{14}C]serotonin by the unstirred platelets did not occur when ADP or epinephrine were the stimulating agents but did occur when the platelets were stimulated with thrombin. At the completion of the incubation, platelet suspensions were layered on 0.5 ml of a mixture of Hi-Phenyl silicone and Methyl silicone oil (4:1) in a 1.5-ml conical centrifuge tube. Free and platelet-bound labeled fibrinogen were separated by centrifugation of the platelets through the silicone oil mixture at 15,600 *g* for 2 min in an Eppendorf centrifuge (Model 5412, Brinkman Instruments, Inc., Westbury, N. Y.) (17). Sedimentation of the platelets was essentially complete within 15 s of centrifugation. After centrifugation, the supernatant buffer and silicone oil were carefully aspirated off the platelet pellet. The tip of the centrifuge tube that contained the pellet was then sliced from the tube and counted for ^{125}I. The quantity of incubation buffer trapped with the pelleted platelets was measured by adding [^{14}C]inulin to the platelet suspension before centrifugation. After centrifugation the distribution of [^{14}C]inulin between the supernate and the platelet pellet was determined. The tips of the centrifuge tubes were placed in scintillation vials that contained 0.5 ml of Protosol and allowed to stand overnight to dissolve the platelet

pellets. 10 ml of Econofluor was added and the vials were counted for ^{14}C. The amount of [^{14}C]inulin in the platelet pellet corresponded to trapping of <0.5% of the original buffer volume with the pellet.

Studies of platelet aggregation. Platelet aggregation was studied by the method of Born (18) using a Chrono-log single channel aggregometer and recorder (Chrono-log Corp., Havertown, Pa.). Platelet suspensions (0.5 ml) were placed in siliconized cuvettes and were stirred at 1,000 rpm at 37°C. Aggregation was measured as an increase in light transmittance as platelet aggregates formed. When platelet suspensions that contained labeled fibrinogen and Ca^{+2} were stirred in the aggregometer without adding an aggregating agent, no change in light transmittance occurred. Moreover, the addition of an aggregating agent to unstirred platelet suspensions that contained fibrinogen and calcium did not produce visible platelet aggregates.

Statistical analyses for this study were performed with the aid of a Wang Model 500 computer (Wang Laboratories Inc., Lowell, Mass.).

RESULTS

Preparation of ^{125}I-fibrinogen. Preparations of human fibrinogen are composed of molecular species differing in solubility, clottability, and molecular weight (8). This heterogeneity has been attributed to partial degradation of the carboxy-terminal region of the Aα-chain, which increases the solubility of the molecule (19). Thus, to obtain fibrinogen free of partially degraded components and other contaminating plasma proteins, the commercially prepared Kabi fibrinogen was separated into low-solubility and high solubility fractions with 16% saturated ammonium sulfate. The low-solubility fraction was further purified by gel filtration over Sepharose 4B and was used in the ensuing studies. Electrophoresis on polyacrylamide gels in SDS (Fig. 1A) showed that the purified, low-solubility fraction consisted of one major molecular species. Electrophoresis after reduction of disulfide bonds (Fig. 1B) demonstrated three polypeptide chains with M_r of 70,000, 55,000, and 45,000 corresponding to the Aα-, Bβ-, and γ-chains of fibrinogen, respectively (20). There was nearly equal staining intensity of the three chains as would be expected with undegraded fibrinogen (8). When incubated with thrombin, the purified fibrinogen was >96% clottable and had a clotting time of 26±4 s, values identical to those obtained by Lipinska et al (8) using the same fibrinogen fraction and clotting procedures. At a concentration of 200 μg/ml, the low-solubility fibrinogen restored the aggregation response of gel-filtered platelets to ADP and epinephrine. In addition, the purified preparation contained no detectable von Willebrand factor when examined both by immunoelectrophoresis and ristocetin-induced platelet agglutination and contained no detectable factor XIII.

The purified low-solubility fibrinogen was radiolabeled with ^{125}I by the iodine-monochloride technique

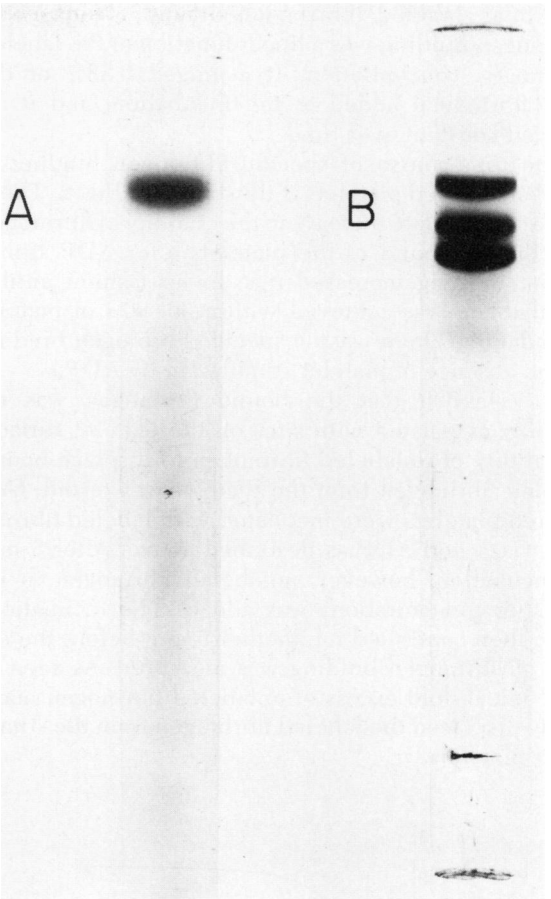

FIGURE 1 SDS-polyacrylamide gel electrophoresis of purified, low-solubility fibrinogen. Low-solubility fibrinogen was dissolved in 1% SDS with or without 0.2 M dithiothreitol and was subjected to disc gel electrophoresis as described in Methods. Protein bands were visualized with 0.1% Coomassie brilliant blue. (A) 3.5% gel, 10 μg of nonreduced fibrinogen. (B) 7.5% gel, 20 μg of reduced fibrinogen.

such that the specific activity of each preparation was ≅2 Ci/mmol. Radiolabeling by this technique did not effect the clottability of the fibrinogen nor its ability to support platelet aggregation.

Binding of ^{125}I-fibrinogen to platelets. Suspensions of 5×10^7 gel-filtered platelets were mixed with 0.5 mM Ca^{++} and ^{125}I-fibrinogen at 150–200 μg/ml and were equilibrated to 37°C. At zero time, 10 μM ADP was added, and the incubations were continued for various time intervals before fibrinogen binding to the platelets was measured. Nonspecific fibrinogen binding was defined as that amount of labeled fibrinogen that could not be displaced from the platelets by the addition of a 10-fold excess of unlabeled fibrinogen. As described further below, this quantity of unlabeled fibrinogen was shown to maximally displace bound labeled fibrinogen. Specific fibrinogen binding was calculated by subtracting the nonspecific binding from

the total labeled fibrinogen bound. Nonspecific fibrinogen binding was a linear function of the labeled fibrinogen concentration. It averaged 0.33% of the total fibrinogen added to the incubation, and it remained constant over time.

The time-course of specific fibrinogen binding to ADP-stimulated platelets is illustrated in Fig. 2. There was an immediate increase in the quantity of fibrinogen specifically bound to the platelets after ADP stimulation. Binding increased in a linear fashion until a steady state was achieved within 60–90 s of platelet stimulation. There was no specific fibrinogen binding in the absence of platelet stimulation by ADP.

To establish that the bound fibrinogen was reversibly associated with sites on the platelet surface, the ability of unlabeled fibrinogen to displace bound labeled fibrinogen from the platelet was tested. Gel-filtered platelets were incubated with labeled fibrinogen, Ca^{+2}, and ADP as described above. After 3 min of incubation, however, unlabeled fibrinogen in increasing concentrations was added. The incubations were then continued for another 5 min before the extent of fibrinogen binding was measured. As seen in Fig. 3, a 10-fold excess of unlabeled fibrinogen maximally displaced the labeled fibrinogen from the stimulated platelets.

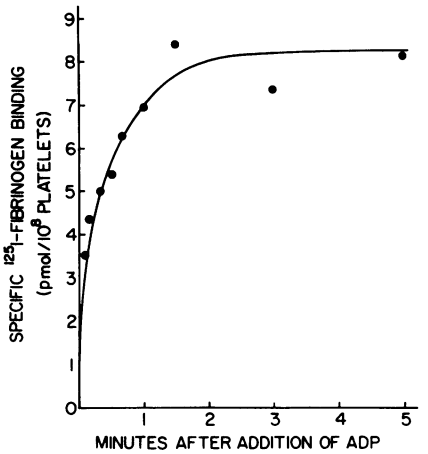

FIGURE 2 Time-course of ^{125}I-fibrinogen binding to ADP-stimulated platelets. Suspensions of 5×10^7 gel-filtered platelets in Tyrode's buffer, pH 7.4, that contained 3.5 mg/ml albumin and 5.5 mM glucose were incubated at 37°C with ^{125}I-fibrinogen (180 μg/ml) and 0.5 mM $CaCl_2$. At zero time, 10 μM ADP was added, and the incubations were continued without stirring for various time intervals. Incubations were terminated by centrifugation of the platelets through silicone oil as described in Methods. Nonspecific fibrinogen binding was measured by performing the binding assays in the presence of 4 mg/ml unlabeled fibrinogen and was subtracted from the total ^{125}I-fibrinogen associated with the centrifuged platelets. In this experiment, nonspecific binding accounted for 37% of the maximum fibrinogen bound. Each point is the mean of triplicate determinations.

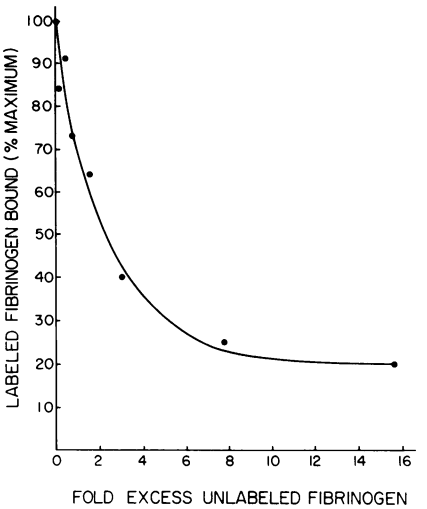

FIGURE 3 Displacement of platelet bound ^{125}I-fibrinogen by unlabeled fibrinogen. Suspensions of 5×10^7 gel-filtered platelets were incubated with ^{125}I-fibrinogen (200 μg/ml), 0.5 mM $CaCl_2$, and 10 μM ADP for 3 min at 37°C without stirring. Unlabeled fibrinogen in increasing concentrations was then added, and the incubations were continued for 5 additional min. The incubations were terminated as described in Fig. 2. In this experiment, nonspecific fibrinogen binding was defined as that labeled fibrinogen that could not be displaced from the platelet and amounted to 20% of the maximum fibrinogen bound. Each point is the mean of triplicate determinations.

To insure that the radioactivity associated with the stimulated platelets represented intact fibrinogen, the radioactive material bound to the platelets was examined by disc gel electrophoresis in SDS. After sedimentation of the stimulated platelets through silicone oil, the platelet pellet was dissolved in 1% SDS, 8 M urea, and 4 mM EDTA by heating at 37°C for 2 h. The dissolved material was then applied to 3.5% disc gels. After electrophoresis, the gels were sliced into 1.5-mm slices and the slices were counted for ^{125}I. Gels that contained only radiolabeled fibrinogen or standard proteins were run concurrently. The radioactivity associated with the stimulated platelets resolved as a major peak whose mobility coincided with that of the radiolabeled fibrinogen (Fig. 4). A small peak of radioactivity with a mobility consistent with that of fibrinogen dimers was present in the dissolved platelets but constituted only 4–6% of the bound material.

Effect of ADP concentration on fibrinogen binding. To determine the effect of ADP concentration on the extent of fibrinogen binding, platelet suspensions that contained 0.5 mM Ca^{+2} and 200 μg/ml labeled fibrinogen were incubated with increasing concentrations of ADP. The extent of fibrinogen binding was then measured after a 3-min incubation. Fibrinogen binding increased in a linear manner as the ADP concentra-

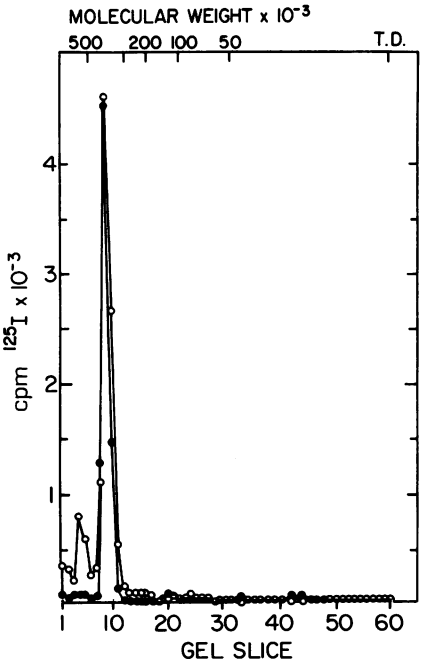

FIGURE 4 SDS-polyacrylamide gel electrophoresis of the ^{125}I-labeled material associated with ADP-stimulated platelets. Suspension of 5×10^7 gel-filtered platelets were incubated with ^{125}I-fibrinogen and 10 μM ADP for 3 min at 37°C as described in Fig. 2. After sedimentation of the labeled platelets through silicone oil, the platelets were dissolved in 1% SDS, 8 M urea, and 4 mM EDTA by heating at 37°C for 2 h and subjected to disc gel electrophoresis as described in Methods. The gels were then sliced into 1.5-mm slices and the slices were counted for ^{125}I. Gels that contained only radiolabeled fibrinogen were run and sliced concurrently. Recovery of the radioactivity applied to the gels was essentially 100%. ○, distribution of ^{125}I associated with ADP-stimulated platelets; ●, ^{125}I-radiochromatogram of radiolabeled fibrinogen; T.D., tracking dye.

tion increased from 0.1 to 1–2 μM, after which the extent of binding reached a plateau (Fig. 5). Thus, these studies demonstrated that fibrinogen binding was directly related to the degree of platelet stimulation by ADP, even at ADP concentrations that produce little if any macroscopic platelet aggregation.

Effect of Ca^{+2} and Mg^{+2} concentration on fibrinogen binding. Fibrinogen binding to ADP-stimulated platelets requires the presence of divalent cations and will not occur in the presence of EDTA. To determine the effect of divalent cation concentration on the extent of fibrinogen binding, suspensions of gel-filtered platelets were incubated with ^{125}I-fibrinogen at 200 μg/ml, 10 μM ADP, and increasing concentrations of either calcium or magnesium chloride for 3 min at 37°C. Maximal steady-state fibrinogen binding was achieved in the presence of 0.5 mM Ca^{+2} or 2.5 mM Mg^{+2} (Fig. 6). Because the extent of fibrinogen binding appeared to decline at concentrations >5 mM Ca^{+2} and 10 mM Mg^{+2},

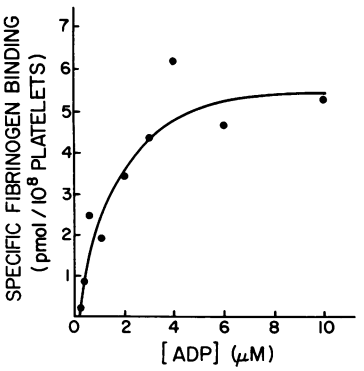

FIGURE 5 Effect of ADP concentration on the extent of ^{125}I-fibrinogen binding. Suspensions of 5×10^7 gel-filtered platelets were incubated with ^{125}I-fibrinogen (200 μg/ml), 0.5 mM $CaCl_2$, and increasing concentrations of ADP for 3 min at 37°C. Specific fibrinogen binding was then determined as described in Fig. 2. Each point is the mean of triplicate determinations.

all subsequent studies were performed in the presence of 0.5 mM Ca^{+2}.

Effect of fibrinogen concentration on steady-state fibrinogen binding. The ability of fibrinogen to saturate the binding sites exposed by ADP was examined by incubating suspensions of gel-filtered platelets with increasing concentrations of labeled fibrinogen, 0.5 mM Ca^{+2}, and 10 μM ADP for 3 min at 37°C (Fig. 7). As the concentration of labeled fibrogen was increased from 0 to 280 μg/ml the quantity of fibrinogen specifi-

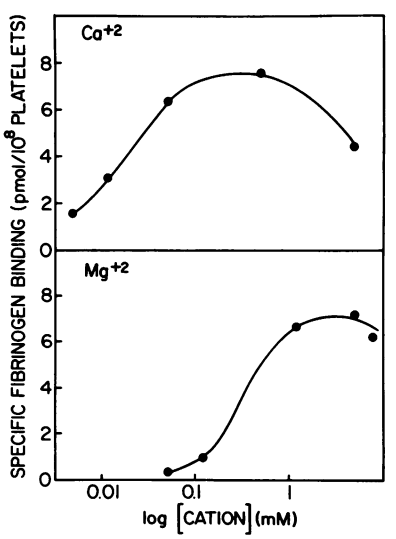

FIGURE 6 Effect of Ca^{+2} and Mg^{+2} concentration on the extent of ^{125}I-fibrinogen binding. Suspensions of 5×10^7 gel-filtered platelets were incubated with ^{125}I-fibrinogen (200 μg/ml), 10 μM ADP, and increasing concentrations of either $CaCl_2$ or $MgCl_2$ for 3 min at 37°C. Specific fibrinogen binding was then determined as described in Fig. 2. Each point is the mean of triplicate determinations.

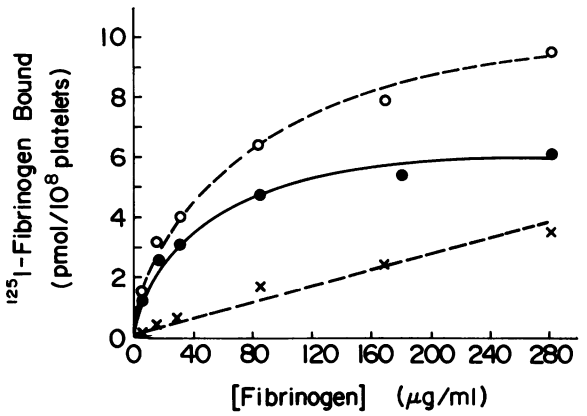

FIGURE 7 Effect of increasing fibrinogen concentration on steady-state fibrinogen binding. Suspensions of 5×10^7 gel-filtered platelets were incubated with increasing concentrations of ^{125}I-fibrinogen, 0.5 mM $CaCl_2$, and 10 μM ADP for 3 min at 37°C. Total, nonspecific, and specific fibrinogen binding were determined as described in Fig. 2. Each point is the mean of triplicate determinations. ○, total ^{125}I-fibrinogen found; ×, nonspecific fibrinogen binding; ●, specific fibrinogen binding.

cally associated with platelets increased toward a plateau value, which suggested the saturation of a finite number of binding sites. When the data were analyzed by the method of Scatchard (21), a straight line was obtained with a correlation coefficient of 0.99 (Fig. 8). Thus, after platelet stimulation by ADP, fibrinogen appears to bind to a uniform population of receptors. Repeating these experiments with platelets from seven different donors indicated that there were 44,000±6,000 (SEM) potential fibrinogen binding sites per platelet with a dissociation constant (K_d) of $8.09 \pm 1.20 \times 10^{-8}$ M or 27±4.1 μg fibrinogen/ml.

The validity of the dissociation constant derived from the equilibrium binding studies was tested in two ways. First, a dissociation constant was calculated from the reaction kinetics according to the formula: K_d = dissociation rate constant (K_2)/association rate constant (K_1) (22). The K_2 was determined by measuring the rate of displacement of labeled fibrinogen from the platelet by a 10-fold excess of unlabeled fibrinogen and equaled 1.08 min^{-1}. The $t_{1/2}$ for fibrinogen displacement was 39 s. The value for K_1 was determined by measuring the rate of specific fibrinogen binding at a variety of fibrinogen concentrations and was equal to 8.49×10^6 M^{-1} min^{-1}. The K_d as determined from the kinetic experiments was 14.3×10^{-8} M, in excellent agreement with the value determined from the equilibrium binding studies. Next, an inhibition constant (K_i) for unlabeled fibrinogen was calculated by determining the concentration of unlabeled fibrinogen that would inhibit labeled fibrinogen binding by 50% (23). The K_i for unlabeled fibrinogen was 11.7×10^{-8} M, again in agreement with the K_d for labeled fibrinogen. Furthermore, this K_i value supports the contention that the fibrinogen was not significantly altered during the radioiodination procedure or as a consequence of platelet binding.

Fibrinogen binding after platelet stimulation by epinephrine. Because fibrinogen is also required for the aggregation of gel-filtered platelets by epinephrine, the effect of epinephrine stimulation on the binding of fibrinogen to platelets was measured. Suspensions of gel-filtered human platelets were mixed with increasing concentrations of labeled fibrinogen and 0.5 mM Ca^{+2}. The binding reaction was initiated by 10 μM epinephrine, and the extent of fibrinogen binding was measured after a 3-min incubation at 37°C. Data were analyzed by the method of Scatchard (21). As illustrated in Fig. 9, platelet stimulation by epinephrine exposed a uniform population of fibrinogen binding sites. Experiments with six different platelet samples indicated that epinephrine stimulation ex-

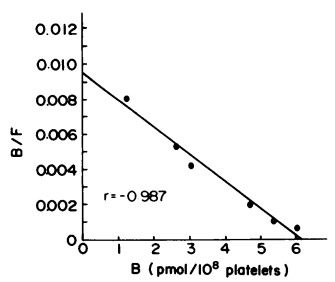

FIGURE 8 Scatchard analysis of ADP-stimulated fibrinogen binding. The data from Fig. 7 were reanalyzed by the method of Scatchard (21). The line represents a linear regression by least squares of the data points. B, platelet bound ^{125}I-fibrinogen; F, concentration of free ^{125}I-fibrinogen.

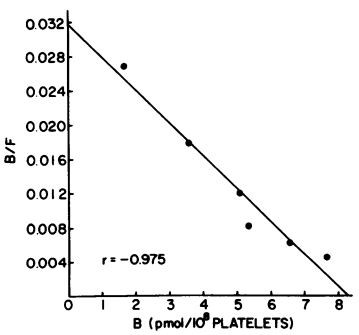

FIGURE 9 Scatchard analysis of epinephrine-stimulated fibrinogen binding. Suspensions of 5×10^7 gel-filtered platelets were incubated with increasing concentrations of ^{125}I-fibrinogen, 0.5 mM $CaCl_2$, and 10 μM epinephrine for 3 min at 37°C. Specific fibrinogen binding was determined as described in Fig. 2. The data were then analyzed by the method of Scatchard (21). Each point represents the mean of triplicate determinations. B, platelet bound ^{125}I-fibrinogen; F, concentration of free ^{125}I-fibrinogen.

posed 48,500±7,000 fibrinogen binding sites per platelet with a K_d of $17.8 \pm 1.79 \times 10^{-8}$ M. These values are essentially the same as those obtained when platelets were stimulated with ADP.

Fibrinogen binding to thrombasthenic platelets. Glanzmann's thrombasthenia is a hemorrhagic disorder in which stimulated platelets undergo shape change and the release reaction but will not aggregate (24). Recently, the membranes of thrombasthenic platelets were shown to be deficient in one and probably two glycoproteins (25). In an attempt to identify tentatively the platelet membrane fibrinogen receptor, the extent of fibrinogen binding to platelets from three patients with thrombasthenia was measured. The binding assays were performed as described above. As seen in Fig. 10, and in contrast to a simultaneously studied normal control, there was essentially no ADP-stimulated specific fibrinogen binding to thrombasthenic platelets. Thus, these studies suggest that the membranes of thrombasthenic platelets may lack the fibrinogen binding sites.

DISCUSSION

The demonstration of fibrinogen receptors on the surface of stimulated platelets suggests that the interaction between fibrinogen and these receptors may account for the role of fibrinogen in platelet aggregation. The validity of this hypothesis, however, requires the demonstration that the fibrinogen-receptor interaction correlates with the established physiology of platelet aggregation (26).

The affinity of the fibrinogen receptor is in agreement with the fibrinogen requirements for platelet function both in vivo and in vitro. Optimal ADP-induced platelet function requires an extracellular fibrinogen concentration of 100–200 μg/ml (1). Saturation of the available fibrinogen binding sites occurs at the same fibrinogen concentrations. Moreover, the affinity of the fibrinogen receptors is consistent with the level of platelet function seen in patients congenitally deficient in fibrinogen. The plasma fibrinogen concentrations of these patients are in the range of 10–50 μg/ml (1, 27). These levels of plasma fibrinogen encompass the K_d of the fibrinogen receptor and thus permit ≅50% of maximal fibrinogen binding. Thus, our data offers an explanation for the relatively mild defects in platelet function seen in this disease (1).

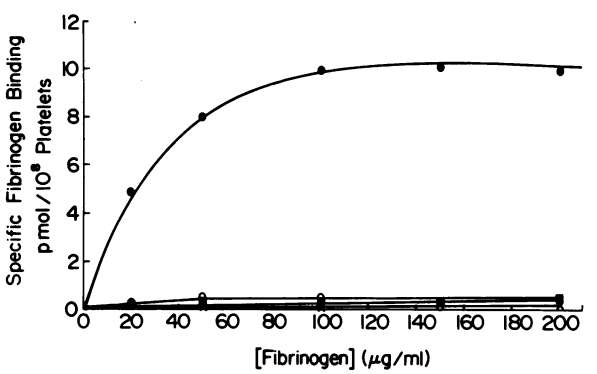

FIGURE 10 Comparison of ADP-stimulated fibrinogen binding to normal and thrombasthenic platelets. Gel-filtered platelets from one normal subject and from three patients with Glanzmann's thrombasthenia were incubated with increasing concentrations of ^{125}I-fibrinogen, 0.5 mM $CaCl_2$, and 10 μM ADP for 3 min at 37°C. Specific fibrinogen binding was determined as described in Fig. 2. Each point represents the mean of triplicate determinations. ●, normal subject; ○, patient 1; ×, patient 2; ■, patient 3.

Platelet function, whether measured in vivo as the bleeding time or in vitro as platelet aggregation, is complete within minutes of platelet stimulation (7, 28). The kinetics of fibrinogen binding are quite compatible with this time frame. For example, at concentrations of fibrinogen barely sufficient to saturate the receptors, steady-state binding is achieved within 60–90 s. Furthermore, at the usual plasma fibrinogen concentrations, which are at least 10-fold greater than the minimal saturating concentrations, half-maximal binding can be calculated to occur within 2 s.

The rapidity with which fibrinogen binds to stimulated platelets suggests a relationship of this binding to the shape change that usually accompanies platelet stimulation. Resolution of this problem is limited by the insensitivity of the platelet aggregometer to the formation of small platelet aggregates. However, fibrinogen binding is a linear function of ADP concentration, even at ADP concentrations that produce only a shape-change response in the aggregometer. Moreover, fibrinogen binding is stimulated by epinephrine, an agent whose aggregation response is not preceded by platelet shape change (29). Our data suggest that the membrane events that result in the exposure of fibrinogen receptors do not require a prior change in platelet shape and that platelet shape change and fibrinogen binding are concurrent but independent consequences of platelet stimulation.

Although the kinetics and affinity of fibrinogen binding correlate with the fibrinogen requirement for platelet aggregation, it is still possible that fibrinogen binding is a secondary rather than a primary event in platelet aggregation. Several of the plasma coagulation factors, including fibrinogen, have been detected on the surface of isolated platelets (7). Controversy exists over the quantity of fibrinogen normally adsorbed to unperturbed platelets (30). On the other hand, because an extracellular source of fibrinogen must be supplied to gel-filtered platelets for ADP- and epinephrine-induced aggregation to occur, it would appear that adsorbed fibrinogen is not capable of supporting aggregation. It is possible, however, that enough

adsorbed fibrinogen was removed from the platelet during gel-filtration to prevent aggregation. The addition of fibrinogen to the platelet suspensions would then permit sufficient readsorption of fibrinogen to restore aggregation. Our data is not consistent with this formulation for several reasons. First, there was no specific fibrinogen binding without prior platelet stimulation by ADP or epinephrine. Second, the extent of specific fibrinogen binding varied directly with the degree of stimulation. Finally, the amount of nonspecifically associated fibrinogen remained constant over time and was consistent with the volume of trapped buffer in the assay system. Thus, our data strongly suggest that it is the fibrinogen bound to the platelet surface as a consequence of platelet stimulation that serves as the cofactor for platelet aggregation.

The mechanism by which receptor-bound fibrinogen may be involved in platelet aggregation remains speculative. Bound fibrinogen may reduce the platelet surface charge sufficiently to allow sites on adjacent platelets to interact. In this regard, Seaman and Vassar (31) found that the platelet surface charge as measured by platelet electrophoretic mobility was uneffected by ADP stimulation when platelets were suspended in saline but was decreased by 15% when platelets were suspended in plasma. Furthermore, a change in platelet surface charge has recently been proposed as a partial explanation for the role of ristocetin in supporting the agglutination of platelets by von Willebrand factor (32). A second possible mechanism is that the large, dimeric fibrinogen molecule may bind to exposed receptors on adjacent platelets and cross-link them. This mechanism has been proposed by Hawiger et al. (33) for the clumping of staphylococci by fibrinogen. However, in contrast to staphylococcal clumping, but in common with platelet aggregation, the binding of fibrinogen to platelets requires the presence of calcium or magnesium ions. In addition, fibrinogen binding appears to be optimal in a divalent cation concentration range that includes the Ca^{+2} concentration of both citrated and noncitrated plasma (34). Because both the platelet surface (35) and the fibrinogen molecule (36) appear to bind calcium, it is possible that the binding of fibrinogen to the platelet involves the formation of intermediary metal ion bridges.

Recently Niewiarowski et al. (37) examined the ability of intact fibrinogen and a variety of degraded fibrinogen species to support platelet aggregation. They noted that both intact carboxy-terminal regions of the Aα-chain and intact amino-terminal regions of the Bβ-chain were required for optimal platelet aggregation. The presence of intact Aα-chains appeared to be of greater importance, however, because the minor degree of Aα-chain degradation that is present in high-solubility fibrinogen was sufficient to decrease its ability to support platelet aggregation by 50%. These findings are of particular interest in view of the suggestion by Marguerie (38) that one of the calcium binding sites on the fibrinogen molecule may be located at the carboxy-terminal end of the Aα-chain.

The failure of stimulated thrombasthenic platelets to bind fibrinogen suggests an explanation for the failure of these platelets to aggregate and further suggests that the surface of these platelets lacks the fibrinogen receptor. The membrane of thrombasthenic platelets has been found to be deficient in two separate glycoproteins with M_r of $\cong$120,000 and 100,000, depending upon the polyacrylamide gel system used for their resolution (25). Degos et al. (39), studying the serum of a patient with thrombasthenia, discovered an antibody that inhibited the aggregation of normal platelets by ADP, epinephrine, thrombin, and collagen, and which reacted with a platelet membrane protein with an M_r of 120,000. Subsequent studies by Levy-Toledano et al. (40) demonstrated that the same antibody did not interfere with ADP-induced platelet shape change. Thus, they suggested that the platelet membrane protein with which the antibody reacted was directly involved in the aggregation phase of platelet function. Because fibrinogen binding also appears to be directly involved in platelet aggregation, it is likely that one of the glycoproteins missing from the thrombasthenic platelet membrane is the platelet fibrinogen receptor.

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REFERENCES

1. Weiss, H. J., and J. Rogers. 1971. Fibrinogen and platelets in the primary arrest of bleeding. *N. Engl. J. Med.* **285:** 369–374.
2. Broekman, M. J., R. I. Handin, and P. Cohen. 1975. Distribution of fibrinogen, and platelet factors 4 and XIII in subcellular fractions of human platelets. *Br. J. Haematol.* **31:** 51–55.
3. Tollefsen, D. M., and P. W. Majerus. 1975. Inhibition of human platelet aggregation by monovalent antifibrinogen antibody fragments. *J. Clin. Invest.* **55:** 1259–1268.
4. Mustard, J. F., M. A. Packham, R. L. Kinlough-Rathbone, D. W. Perry, and E. Regoeczi. 1978. Fibrinogen and ADP-induced platelet aggregation. *Blood.* **52:** 453–466.
5. Tangen, O., E. L. McKinnon, and H. J. Berman. 1973. On the fine structure and aggregation requirements of gel filtered platelets. *Scand. J. Haematol.* **10:** 96–105.
6. Miller, J. L., A. J. Katz, and M. B. Feinstein. 1975. Plasmin inhibition of thrombin-induced platelet aggregation. *Thromb. Diath. Haemorrh.* **33:** 286–309.

7. Weiss, H. J. 1975. Platelet physiology and abnormalities of platelet function. *N. Engl. J. Med.* **293:** 531–540, 580–588.
8. Lipinska, I., B. Lipinski, and V. Gurewich. 1974. Fibrinogen heterogeneity in human plasma. Electrophoretic demonstration and characterization of two major fibrinogen components. *J. Lab. Clin. Med.* **84:** 509–516.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
10. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
11. Allain, J. P., H. A. Cooper, R. H. Wagner, and K. M. Brinkhous. 1975. Platelets fixed with paraformaldehyde: a new reagent for assay of von Willebrand factor and platelet aggregating factor. *J. Lab. Clin. Med.* **85:** 318–328.
12. Zimmerman, T. S., O. D. Ratnoff, and A. E. Powell. 1971. Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand disease. *J. Clin. Invest.* **50:** 244–254.
13. Loewy, A. S., D. Dunathan, and R. Kriel. 1960. Fibrinase. I. Purification of substrate and enzyme. *J. Biol. Chem.* **236:** 2625–2633.
14. Izzo, J. L., W. F. Bale, M. J. Izzo, and A. Roncone. 1964. High specific activity labeling of insulin with ^{131}I. *J. Biol Chem.* **239:** 3743–3748.
15. Tangen, O., H. J. Berman, and P. Marfey. 1971. Gel filtration–a new technique for separation of blood platelets from plasma. *Thromb. Diath. Haemorrh.* **25:** 268–278.
16. Jerushalmy, Z., and M. B. Zucker. 1966. Some effects of fibrinogen degradation products (FDP) on blood platelets. *Thromb. Diath. Haemorrh.* **15:** 413–419.
17. Feinberg, H., H. Michel, and G. V. R. Born. 1974. Determination of the fluid volume of platelets by their separation through silicone oil. *J. Lab. Clin. Med.* **84:** 926–934.
18. Born, G. V. R. 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature (Lond.).* **194:** 927–929.
19. Galanakis, D. K., M. W. Mosesson, and N. E. Stathakis. 1978. Human fibrinogen heterogeneities: distribution and charge characteristics of chains of Aα origin. *J. Lab. Clin. Med.* **92:** 376–386.
20. Doolittle, R. F. 1977. Structure and function of fibrinogen. *Horiz. Biochem. Biophys.* **3:** 164–191.
21. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51:** 660–672.
22. Rodbard, D., and H. A. Feldman. 1975. Theory of protein-ligand interaction. *Methods Enzymol.* **36:** 3–16.
23. Cheng, Y. C., and W. H. Prusoff. 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22:** 3099–3108.
24. Caen, J. 1972. Glanzmann thrombasthenia. *Clin. Haematol.* **1:** 383–392.
25. Phillips, D. R., and P. P. Agin. 1977. Platelet membrane defects in Glanzmann's thrombasthenia. *J. Clin. Invest.* **60:** 535–545.
26. Catt, K. J., and M. L. Dufau. 1977. Peptide hormone receptors. *Annu. Rev. Physiol.* **39:** 529–557.
27. Gralnick, H. R. 1977. Congenital disorders of fibrinogen. *In* Hematology. W. J. Williams, E. Beutler, A. J. Erslev, and R. W. Rundles, editors. McGraw-Hill Book Co., New York. 2nd edition. **156:** 1423–1429.
28. Harker, L. A., and S. J. Slichter. 1972. The bleeding time as a screening test for evaluation of platelet function. *N. Engl. J. Med.* **287:** 155–159.
29. O'Brien, J. R. 1965. Effects of adenosine diphosphate and adrenaline on mean platelet shape. *Nature (Lond.).* **207:** 306–307.
30. Zucker, M. B. 1977. Relationship of plasma clotting factors to platelets. *In* Topics in Hematology. S. Seno, F. Takaku, and S. Irino, editors. Excerpta Medica, Amsterdam. 280–282.
31. Seaman, G. V. F., and P. S. Vassar. 1966. Changes in the electrokinetic properties of platelets during their aggregation. *Arch. Biochem. Biophys.* **117:** 10–17.
32. Coller, B. S. 1978. The effects of ristocetin and von Willebrand factor on platelet electrophoretic mobility. *J. Clin. Invest.* **61:** 1168–1175.
33. Hawiger, J., D. K. Hammond, S. Timmons, and A. Z. Budzynski. 1978. Interaction of human fibrinogen with staphylococci: presence of a binding region on normal and abnormal fibrinogen variants and fibrinogen derivatives. *Blood.* **51:** 799–812.
34. Holmsen, H. 1972. The platelet: its membrane, physiology, and biochemistry. *Clin. Haematol.* **1:** 235–266.
35. Bennett, J. S., R. F. Colman, and R. W. Colman. 1978. Identification of adenine nucleotide binding proteins in human platelet membranes by affinity labeling with 5′-*p*-fluorosulfonylbenzoyl adenosine. *J. Biol. Chem.* **253:** 7346–7354.
36. Marguerie, G., G. Chagniel, and M. Suscillon. 1977. The binding of calcium to bovine fibrinogen. *Biochim. Biophys. Acta.* **490:** 94–103.
37. Niewiarowski, S., A. Z. Budyznski, and B. Lipinski. 1977. Significance of the intact polypeptide chains of human fibrinogen in ADP-induced platelet aggregation. *Blood.* **49:** 635–644.
38. Marguerie, G. 1977. The binding of calcium to fibrinogen: some structural features. *Biochim. Biophys. Acta.* **494:** 172–181.
39. Degos, L., A. Dautigny, J. C. Brouet, M. Colombani, N. Ardaillou, J. P. Caen, and J. Colombani. 1975. A molecular defect in thrombasthenic platelets. *J. Clin. Invest.* **56:** 236–240.
40. Levy-Toledano, S., G. Tobelem, C. Legrand, R. Bredoux, L. Degos, A. Nurden, and J. P. Caen. 1978. Acquired IgG antibody occurring in a thrombasthenic patient: its effect on human platelet function *Blood.* **51:** 1065–1071.