Normal primary platelet aggregation requires agonist-mediated activation of membrane GPIIb-IIIa, binding of fibrinogen to GPIIb-IIIa, and cellular events after ligand binding. PAC1 monoclonal antibody distinguishes between resting and activated states of GPIIb-IIIa, and other antibodies preferentially recognize GPIb (PMI-1) or IIIa (anti-LIBS1) after the binding of fibrinogen or fibrinogen-mimetic peptides, such as GRGDSP. Using these antibodies and platelet flow cytometry, we studied two distinct persistent platelet aggregation abnormalities. Platelets from a thrombasthenic variant, which contained near-normal amounts of GPIIb-IIIa, failed to aggregate or bind PAC1 in response to agonists. In addition, GRGDSP, which binds to normal GPIIIb-IIIa without prior cell activation, failed to increase the binding of PMI-1 or anti-LIBS1 to the thrombasthenic platelets, suggesting a primary defect in ligand binding.

PRIMARY PLATELET aggregation is essential for normal hemostasis and also plays a role in thrombosis. This process is dependent on the binding of fibrinogen to platelet membrane glycoprotein (GP) IIb-IIIa. Efficient binding of large GPIIb-IIIa ligands, such as fibrinogen or the anti–GPIIb-IIIa monoclonal antibody (MoAb) PAC1, requires platelet activation by agonists such as ADP or thrombin. In contrast, small fibrinogen-mimetic ligands, such as Arg-Gly-Asp (RGD)–containing synthetic peptides, can bind to GPIIb-IIIa in an activation-independent manner. This had led to the hypothesis that the ligand-binding pocket of GPIIIb-IIIa is present on resting cells, but it is of limited accessibility to large ligands such as fibrinogen and PAC1. Agonist-induced expression of the fibrinogen receptor on GPIIb-IIIa appears to involve G protein–mediated activation of phospholipase C, followed by activation of protein kinase C. Under some conditions, other metabolic pathways are also involved, but their identity remains to be determined, as do the nature of the changes in GPIIb-IIIa that render it competent to bind fibrinogen.

In addition to fibrinogen receptor exposure and ligand binding, events subsequent to fibrinogen binding appear to be necessary for maximum platelet aggregation. One such postoccupancy event involves the GPIIb-IIIa complex itself—conformational changes that can be detected by antibodies that bind preferentially to GPIIb or IIIa when the fibrinogen receptor is occupied, either by fibrinogen or by an RGD-containing peptide. One of these conformational changes is detectable with the anti–GPIIb antibody PMI-1, and another change is detectable with the anti–GPIIIa antibody anti-LIBS1. It is not yet clear how postoccupancy events influence the extent of platelet aggregation. Of note in this regard, however, the GPIIb-IIIa complex has been implicated in stimulus-response coupling and in platelet calcium homeostasis. Moreover, the binding of fibrinogen to platelets induces conformational changes in the ligand itself, thereby possibly augmenting its adhesive function. The three processes involving GPIIb-IIIa that are necessary for platelet aggregation—activation, ligand binding, and postoccupancy events—are illustrated schematically in Fig 1.

The most profound defects in platelet aggregation occur in patients with the autosomal-recessive hereditary disorder, Glanzmann’s thrombasthenia. Although the platelets of most homozygotes with the classic form of this disease possess less than 10% GPIIb-IIIa, platelets from some functional variants possess near normal levels of GPIIb-IIIa. Accordingly, these latter individuals may have a defect involving activation, ligand binding, or postoccupancy functions of GPIIb-IIIa. It is likely that abnormalities in one or more of these three processes are also involved in the broad spectrum of acquired aggregation defects in such clinical settings as myeloproliferative disorders, drug administration, uremia, and postcardiopulmonary bypass.

Recent advances in flow cytometry have made it possible to rapidly study the quantity and function of membrane
proteins on intact platelets. These analyses can be performed on small samples of blood or platelet-rich plasma. In the present study, we have used this approach to develop a strategy to characterize platelet aggregation abnormalities in pathophysiologic terms as defects in either GPIIb-IIIa activation, ligand binding, or postoccupancy events. GPIIb-IIIa activation and ligand binding were assessed with the antibody PAC1, whereas postoccupancy events were assessed with the antibodies PMI-1 and anti-LIBS1. The advantages of this approach are illustrated by analysis of a functional variant of thrombasthenia, which we now report is due to a defect in ligand recognition. As a further example, we show that the platelets from an individual with myocardial infarction and an acquired aggregation disorder exhibit an agonist-specific defect in activation of GPIIb-IIIa.

METHODS

Antibodies. Preparation and characterization of the antibodies used here have been described in detail elsewhere and their properties are listed in Table 1. Tab and 4F10 were generously provided by Dr Rodger McEver, Oklahoma City, OK, and Dr Virgil Woods, UCSD Medical Center, San Diego, CA, respectively. 10E5 and 7E3 were the generous gifts of Dr Barry Coller, SUNY, Stony Brook, NY. Fluorescein isothiocyanate-conjugated antibodies were prepared as described to achieve fluorescein/protein molar ratios of 3 to 6.

Peptides. Peptides were synthesized in a central facility at the Research Institute of Scripps Clinic using an Applied Biosystems (Mountain View, CA) peptide synthesizer. Peptides were more than 90% homogeneous by high performance liquid chromatography, and amino acid compositions were consistent with the desired sequence. The peptides were Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), fibrinogen γ130,131 (Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val), and Lys-Tyr-Gly-Arg-Gly-Asp-Ser (KYGRGDS).

Analysis of platelet surface GPIIb-IIIa by flow cytometry. Platelet-rich plasma was obtained by taking venous blood from patients and controls who had not ingested medications for at least 10 days, anticoagulating with 1/10 volume of 3.8% sodium citrate, and sedimenting red and white blood cells at 180g for 20 minutes at room temperature. Immediately thereafter, 5-μL aliquots of platelet-rich plasma were added to polypropylene tubes containing a fluorescein isothiocyanate-conjugated monoclonal antibody (10^-5 to 10^-10 mol/L) in Tyrode’s buffer (% bovine serum albumin, 2 mmol/L MgCl2, 137.5 mmol/L NaCl, 12 mmol/L NaHCO3, 2.6 mmol/L KCl, pH 7.4). Samples were incubated without stirring in a total volume of 50 μL for 15 minutes at room temperature with agonists and peptides as indicated. Samples were then diluted to 0.5 mL with Tyrode’s buffer and analyzed on a FACStar flow cytometer (Bectin-Dickinson Immunocytometry Systems, Mountain View, CA). Light scatter and fluorescence signals were acquired at logarithmic gain, and 10,000 platelets in each sample were analyzed.

Results are expressed as mean platelet fluorescence intensity in arbitrary fluorescence units or as histograms of log platelet fluorescence intensity in arbitrary units on the abscissa and platelet number on the ordinate.

Isolation and surface radioiodination of platelets. Platelets were isolated from acid-citrate dextrose anticoagulated human blood by differential centrifugation followed by gel filtration on Sepharose 2B. Lysates of surface-labeled platelets were analyzed by KYGRGDS affinity chromatography as described. Briefly, intact
platelets were radioiodinated by the lactoperoxidase-H₂O₂ method then resuspended to 2 × 10⁹ platelets/mL and solubilized in lysis buffer containing 10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.5, 0.15 mol/L NaCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 0.1 mmol/L leupeptin, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 10 mmol/L N-ethylmaleimide, and 50 mmol/L octylglucoside. The lysates were incubated at 4°C for 12 hours with KYGRGDS coupled to CNBr-activated Sepharose, washed, and eluted with 1 mmol/L GRGDSP. Under these conditions approximately 10% of normal GPIIb-IIIa is bound and eluted. A comparable quantity is bound on rechromatography of the pass through fraction, indicating that less than 100% binding is due to the low affinity of the interaction rather than an inactive subpopulation of GPIb-IIIa.30

Patients. The Cam variant of Glanzmann's thrombasthenia has been described in detail.17 Patients with this variant have near normal quantities of GPIIb-IIIa, undetectable fibrinogen binding to platelets, and absent platelet aggregation. The second patient is a 58-year-old woman who noted increased bruising in 1984; studies showed a platelet count of 1,500,000/μL and a bone marrow with megakaryocytic hyperplasia and myelofibrosis. Since diagnosis, her bruising has been constant, primarily involving her extremities and anterior chest, and she notes occasional epistaxis. She has had four episodes of gastrointestinal bleeding, each requiring transfusion with 2 to 5 U of RBCs. Before the onset of bruising, she had withstood several hemostatic challenges, including tonsillectomy, hemorrhoidectomy, and colecystectomy, as well as three uncomplicated vaginal deliveries. Physical examination at present is normal except for hepatosplenomegaly and generalized bruising. Recent laboratory findings: hematocrit 33, white blood cell count 29,600/μL with 27 neutrophils, 18 bands, 12 metamyelocytes, 11 myelocytes, 8 blasts, 2 monocytes, 21 lymphocytes, 7 basophils, and 6 nucleated RBCs. The patient had an abnormal bleeding time of 20 minutes and a normal conglutination profile (prothrombin time, activated partial thromboplastin time, thrombin time, and fibrin split products) and von Willebrand panel (von Willebrand antigen and ristocetin cofactor activity, and normal von Willebrand multimers distribution). Platelet aggregation studies, performed after receiving no medications, revealed absent aggregation to 10 and 100 μmol/L adenosine diphosphate (ADP), 50 μmol/L epinephrine, and collagen.

RESULTS

Based on the model depicted in Fig 1, abnormal platelet aggregation could arise from a defect in the activation of platelet fibrinogen receptors, a defect in fibrinogen binding per se, or a defect in the postoccupancy events required for optimal aggregation. The potential usefulness of this model was tested by studying two patients with persistent and severe defects in platelet aggregation. To quantitate fibrinogen receptor activation and ligand binding, we used a fluorescently labeled MoAb, PAC1, that specifically recognizes the activated form of GPIIb-IIIa. Antibody binding was measured in small (5 μL) samples of platelet rich plasma by flow cytometry.

The Cam variant of thrombasthenia is due to a defect in ligand recognition. The Cam variant of Glanzmann's thrombasthenia is characterized by markedly reduced fibrinogen binding to platelets that contain near normal quantities of GPIIb-IIIa.17 The presence of GPIIb-IIIa on the Cam platelet surface was confirmed by flow cytometry, in which there was comparable binding of an anti-GPIIb MoAb (Tab) and an anti-GPIIIa MoAb (AB-15) to Cam and normal platelets. In addition, four MoAbs specific for the GPIIb-IIIa complex (A₂A₄, 7E3, 10E5, 4F10), all of which recognize the nonactivated form of GPIIb-IIIa at 1:1 stoichiometry22-26 and inhibit fibrinogen binding to platelets, also bound in comparable amounts to the Cam and normal platelets (Fig 2). In sharp contrast, there was a complete lack of binding of the activation-specific MoAb PAC1 to ADP-activated Cam platelets (Fig 2). This defect was not specific to ADP stimulation, because Cam platelets activated with 50 nmol/L phorbol myristate acetate (PMA) also showed no increase in PAC1 fluorescence (data not shown).

Because PAC1 may recognize the ligand binding site in GPIIb-IIIa,22-26 the absence of PAC1 binding could be due to a lack of GPIIb-IIIa activation, or it could be due to a defect in ligand binding. To distinguish these possibilities, we exploited the observation that small fibrinogen-mimetic peptides bind to GPIIb-IIIa in an activation-independent manner.
manner. The binding of such a peptide, GRGDSP, was assayed by use of PMI-1 and anti-LIBS1 MoAbs, which preferentially recognize GPIIb-IIIa in a 1:1 stoichiometry, following the binding of either fibrinogen or small peptide ligands. Platelets from a normal individual showed a 2.5-fold increase in PMI-1 binding in the presence of 200 μmol/L GRGDSP (mean fluorescence intensity of untreated platelets = 60 arbitrary fluorescence units; GRGDSP-treated platelets = 151 fluorescence units) (Fig 2). In contrast, Cam platelets showed no increase in PMI-1 binding in response to GRGDSP (mean fluorescence intensity of untreated platelets = 102 fluorescence units; GRGDSP-treated platelets = 107 fluorescence units) (Fig 2).

It should be noted that PMI-1 binding to untreated Cam platelets was higher than normal. Therefore, the binding of this antibody could not be used here as an indicator of the binding of GRGDSP to Cam platelets. However, the anti-LIBS1 antibody was more informative in this regard. As shown in Fig 2, untreated Cam platelets bound less anti-LIBS1 than control platelets (mean fluorescence of 27 and 58 U, respectively). The fibrinogen-mimetic peptide, GRGDSP, caused a fourfold increase in anti-LIBS1 binding to normal platelets (mean fluorescence, 205 U); however, no increase in antibody binding was observed in Cam platelets (mean fluorescence, 25 U). Anti-LIBS1 binding to Cam platelets also failed to increase when platelets were treated with another fibrinogen-mimetic peptide YG02-41 (400 μmol/L), instead of GRGDSP (control platelets, 201 fluorescence U; Cam platelets, 31 U). When anti-LIBS1 binding was examined over a range of GRGDSP peptide concentrations, control platelets showed a saturable increase in antibody binding such that half-maximal expression of the LIBS1 epitope on GPIIIa was observed at 20 μmol/L peptide. This is similar to the IC₅₀ of this peptide for fibrinogen binding to activated platelets. Instead, at 200 μmol/L GRGDSP, anti-LIBS1 binding to Cam platelets was less than that seen with control platelets in the presence of 1 μmol/L peptide, suggesting that the affinity of Cam GPIIb-IIIa for this peptide was at least 200-fold less than normal GPIIb-IIIa (Fig 3).

The foregoing data, by use of conformation-specific antibodies and intact cells, indicated that the Cam variant was due to a deficit in ligand recognition by GPIIb-IIIa. To directly examine the ligand binding function of Cam GPIIb-IIIa, we subjected extracts of I²⁵I surface-labeled platelets to affinity chromatography using an immobilized RGD peptide. As illustrated in Fig 4, when surface-labeled normal platelets were passed over a KYGRGDS peptide matrix followed by the GRGDSP peptide, GPIIb and GPIIIa were bound and eluted. In contrast, with Cam platelets no such GPIIb-IIIa was eluted, even though the starting extract contained comparable quantities of surface-labeled GPIIb-IIIa (Fig 4, lane 1). We quantitated the GPIIb-IIIa content in the starting material and in the column fractions by use of a polyclonal anti-GPIIb-IIIa antibody in an ELISA assay. The control extract contained 332 μg; the Cam individual contained 240 μg in the starting extract. The eluted fractions from the control extract contained 20 μg GPIIb-IIIa. In contrast, those from the Cam extract contained 2.6 μg. Thus, Cam GPIIb-IIIa has a deficit in the recognition of ligands such as RGD peptides.

Reduced platelet aggregation in a patient with myelofibrosis is caused by a defect in the activation of GPIIb-IIIa. The work described above established the utility of conformation-specific antibodies and flow cytometry in characterizing a defect in ligand binding. We next applied this strategy to the characterization of a patient with myelofibrosis and an acquired severe defect in platelet aggregation. Platelets from this patient did not express PAC1 binding sites when stimulated with 10 μmol/L ADP (Fig 5) or 50 μmol/L epinephrine (not shown), indicating a defect either in GPIIb-IIIa activation or ligand binding. In contrast to the Cam patient, anti-LIBS1 and PMI-1 signals in the presence and absence of GRGDSP were similar to the normal control (Fig 5), indicating intact ligand binding function. Furthermore, PAC1 bound to the patient's platelets in response to PMA, which circumvents normal receptor-mediated pathways by directly activating protein kinase C. The patient's platelets also exhibited normal α-granule secretion in response to PMA, as evidenced by surface expression of the α-granule protein thrombospondin (Fig 5). The aggregation defect in the patient's platelets could not be reversed by suspending her platelets in normal plasma, and the defect could not be induced in normal platelets by incubation in the patient's plasma. Thus, the GPIIb-IIIa from this patient has the potential to bind RGD ligands, but there appears to be an intrinsic cellular defect in receptor-mediated activation of GPIIb-IIIa.

DISCUSSION

For two decades, studies of platelet aggregation in vitro have been routinely used in the characterization of patients with a prolonged bleeding time or a bleeding diathesis due to platelet dysfunction. The results of recent studies have...
Fig 4. RGD affinity chromatography of Cam versus normal platelet extract. Extracts of surface-labeled Cam and normal platelets were incubated overnight at 4°C with KYGRGDS coupled to CNBr activated Sepharose. After washing, the column was eluted with 1 mmol/L GRGDSP peptide. Starting material is shown in lane 1, column flow through in lane 2, and last wash fraction in lane 3. The GRGDSP elution began in lane 4 and continued to lane 8. Shown are autoradiograms of 7.5% SDS gels of these fractions under nonreducing conditions.

Fig 5. Flow cytometric analysis of aggregation dysfunction in a patient with myelofibrosis. Patient platelets (MPD) are shown in the left hand panels, control platelets in the right. The disclosing antibody is indicated in the left hand column. In each row, the legend indicates the added agonist or peptide. Agonist and peptide concentrations were identical to those in Fig 2. Phorbol myristate acetate (PMA) was present in a final concentration of 50 nmol/L. Anti-TSP refers to the binding of an MoAb against thrombospondin.

demonstrated that normal aggregation requires the binding of fibrinogen to platelet GPIIb-IIIa. Furthermore, the aggregation process may be viewed as a series of necessary cellular events: (a) agonist-induced activation of GPIIb-IIIa, resulting in the exposure of the fibrinogen binding site; (b) fibrinogen binding; and (c) postoccupancy events that follow upon ligand binding (Fig 1). Based on the flow cytometric approach described in this report, it is possible to classify disorders of platelet aggregation in terms of this series of events. The flow cytometry method used here is rapid and can be performed on platelet-rich plasma or on whole blood. This approach has been made possible by the availability of MoAbs that can distinguish between the resting, activated, and ligand-occupied forms of GPIIb-IIIa. The three types of platelet dysfunction that can lead to reduced platelet aggregation and the predicted flow cytometry results are summarized in Table 2.

Using this approach, we have found that a variant of Glanzmann’s thrombasthenia is due to a defect in the ligand-binding function of GPIIb-IIIa and that the markedly reduced aggregation in a patient with myelofibrosis is due to a defect in agonist-specific activation of GPIIb-IIIa. We have not yet identified a patient with postoccupancy dysfunction. However, such a postoccupancy defect may explain the process of agonist-induced platelet desensitization.

Table 2. Flow Cytometric Analysis of Platelet Aggregation Dysfunction

<table>
<thead>
<tr>
<th>Type of Defect</th>
<th>AGG*</th>
<th>PAC1†</th>
<th>LIBS‡</th>
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<tbody>
<tr>
<td>None</td>
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<td></td>
</tr>
<tr>
<td>Activation</td>
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<td>Binding</td>
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<tr>
<td>Postoccupancy</td>
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</table>

Indicated are presence (+) or absence (−) of a given response.

*Agonist-stimulated platelet aggregation.
†Increased binding of activation-dependent ligand such as PAC1 in response to agonist.
‡Increased binding of occupancy-dependent antibody, such as anti-LIBS 1, in the presence of activation-independent ligand, such as GRGDSP.
ple, unstimulated platelets activated for more than 10 minutes with ADP or epinephrine in the presence of fibrinogen demonstrate a marked reduction in their subsequent aggregation response when compared with the same platelets incubated without fibrinogen.\textsuperscript{10,11} This cannot be explained by a reduction in fibrinogen binding. Many patients are encountered in clinical practice with easy bruisability, a prolonged bleeding time, and a decreased platelet aggregation in response to one or more agonists. After excluding aspirin ingestion and storage pool disease, the underlying cause is usually inapparent. This group of individuals is often referred to as having “aspirinlike” defects, and some may have congenital or acquired platelet metabolic defects leading to an abnormality of GPIIb-IIIa activation. It seems likely that among this heterogeneous group of patients, individuals with the postoccupancy dysfunction phenotype will also be identified.

The Cam variant of thrombasthenia is due to a defect in ligand recognition by GPIIb-IIIa. This conclusion is based on (a) the failure of Cam GPIIb-IIIa to bind to an insolubilized RGD peptide and (b) a greater than 200-fold reduction in the capacity of activation-independent peptide ligands to increase the binding of the occupancy-dependent anti-LIBS\textsubscript{1} antibody. Previous studies with proteolytic fragments of fibrinogen and fibrinogen-related peptides had implicated recognition of RGD and fibrinogen gamma chain peptide sequences in fibrinogen binding.\textsuperscript{3,15} In the present study, GPIIb-IIIa lacking the capacity to bind these peptide sequences lacked the capacity to support fibrinogen binding and platelet aggregation. In view of the apparent autosomal-recessive inheritance of the Cam variant,\textsuperscript{7} the severe functional defect in GPIIb-IIIa, and the intermediate functional defect of the parents, it is likely that the basic Cam defect is due to a point mutation in GPIIb-IIIa. Such a point mutation has recently been identified in a Cam patient.\textsuperscript{35} The observation that this single amino acid change leads to loss of binding of both the RGD and gamma chain peptide sequences favors the possibilities that both peptide sequences are recognized by a common binding site.

The PAC1 antibody was used to monitor GPIIb-IIIa activation because it binds selectively and with high affinity to stimulated platelets.\textsuperscript{13} Moreover, based on the inhibition of PAC1 binding by fibrinogen\textsuperscript{37} and fibrinogen-mimetic peptides\textsuperscript{38} and the capacity of peptides derived from the hypervariable region of PAC1 to inhibit fibrinogen binding,\textsuperscript{31} it seems likely that this antibody recognizes the ligand binding site in GPIIb-IIIa. This hypothesis is strongly supported by the finding that PAC1 fails to recognize the Cam mutant GPIIb-IIIa, which lacks ligand binding function. The failure of PAC1 to interact with Cam platelets is unlikely to be due to gross denaturation of GPIIb-IIIa, because four other complex-specific anti-GPIIb-IIIa antibodies appeared to bind to the same extent as anti-GPIIb or anti-GPIIIa antibodies. Of these four antibodies, 7E3 binds more rapidly to activated cells\textsuperscript{35} and A\textsubscript{2}A\textsubscript{3} is inhibited by synthetic peptides derived from the fibrinogen gamma chain.\textsuperscript{34} Because Cam GPIIb-IIIa lacks the capacity to recognize fibrinogen or the peptide ligands, it seems likely that these previous findings are due to an indirect relationship between the 7E3 and A\textsubscript{2}A\textsubscript{3} epitopes and the ligand binding site of GPIIb-IIIa.

GPIIb-IIIa is a member of the integrin family of structurally related adhesion receptors.\textsuperscript{39-42} Included in this family are a platelet collagen receptor,\textsuperscript{43} leukocyte receptors involved in inflammation and defense against pyogenic infection,\textsuperscript{44} and receptors involved in lymphocyte homing.\textsuperscript{45} In the case of the leukocyte receptors, recent studies have reported that activation of these receptors induces leukocyte aggregation and endothelial cell adherence.\textsuperscript{46} Moreover, occupancy-dependent MoAbs have been prepared against an endothelial cell integrin.\textsuperscript{12} This suggests that activation-specific and occupancy-dependent antibodies might be prepared against other integrins and be used to analyze leukocyte dysfunction in a manner analogous to that described here for GPIIIb-IIIa. Indeed, there is no obvious reason to preclude these strategies for the rapid analysis of binding functions of nonintegrin cell surface receptors as well.

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Analysis of platelet aggregation disorders based on flow cytometric analysis of membrane glycoprotein IIb-IIIa with conformation-specific monoclonal antibodies

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