Platelet membrane glycoprotein IIb-IIIa exists as a calcium-dependent complex of two large peptides (designated IIb and IIIa) in Triton X-100 solutions, but it remains unknown if these peptides are subunits of one glycoprotein or are actually two individual glycoproteins in the intact platelet membrane. We used crossed immunoelectrophoresis to define the epitopes of two monoclonal antibodies to IIb-IIIa, then used these antibodies to study the structural and functional organization of IIb and IIIa in the platelet membrane. Human platelets solubilized in Triton X-100 were electrophoresed through an intermediate gel containing $^{125}$I-monoclonal IgG, then into an upper gel containing rabbit anti-human platelet antibodies. Our previously characterized antibody, Tab, and a new monoclonal antibody, $T_10$, both bound to the immunoprecipitate corresponding to the IIb-IIIa complex. When platelets were electrophoresed after solubilization in 5 mM EDTA, $^{125}$I-Tab bound to the dissociated IIb polypeptide, but not to IIIa. In contrast, $^{125}$I-$T_10$ did not react with either IIb or IIIa. Thus, Tab recognizes a determinant on IIb, while $T_10$ recognizes a determinant created only after the association of IIb and IIIa. Gel-filtered platelets from six normal donors bound $5.0 \pm 0.3 \times 10^{10}$ $^{125}$I-$T_10$ molecules/platelet and $47.800 \pm 11.290$ $^{125}$I-Tab molecules/platelet, consistent with IIb-IIIa being a heterodimer. $^{125}$I-$T_10$ binding was identical in unactivated platelets and platelets stimulated with 10 $\mu$M ADP. However, platelets did not aggregate or bind $^{125}$I-fibrinogen until ADP was added. $T_10$, but not Tab or nonimmune mouse antibody, inhibited ADP-induced platelet aggregation and $^{125}$I-fibrinogen binding. Our findings suggest that IIb and IIIa exist as subunits of a single membrane glycoprotein in unstimulated platelets. Fibrinogen binding appears to require not only the interaction of IIb and IIIa, but also some additional change occurring after platelet activation.

Platelets stimulated with ADP, epinephrine, or thrombin expose specific receptors for fibrinogen on their surface, a reaction which appears to be required for normal platelet aggregation (1–5). Evidence for the involvement of membrane glycoproteins in fibrinogen binding and aggregation has been derived from the study of platelets from patients with the inherited bleeding disorder, Glanzmann’s thrombasthenia (6). These platelets do not bind fibrinogen (1, 7, 8) and do not aggregate, abnormalities which appear to be related to the deficiency of membrane glycoproteins known as IIb and IIIa. The experiments reported here provide evidence that IIb and IIIa are polypeptide subunits of a single membrane glycoprotein which together form the platelet membrane receptor for fibrinogen.

We previously reported using a monoclonal antibody, named Tab, to co-isolate both IIb and IIIa from Triton X-100-solubilized platelets by affinity chromatography (9, 10). We assumed that Tab bound either to IIb or IIIa and that these polypeptides were isolated together because they formed a complex in Triton X-100 and may also be associated in the intact platelet membrane. Experiments employing crossed immunoelectrophoresis of Triton X-100-solubilized platelet proteins against rabbit antplatelet antibodies led to similar conclusions, since both IIb and IIIa could be eluted from a single immunoprecipitate (11). Kunicki et al. (12) subsequently employed CIE to show that the association of IIb and IIIa was mediated by calcium ions (12). When platelets were solubilized in EDTA and then electrophoresed, the major IIb-IIIa immunoprecipitate dissociated into two new ars from which IIb and IIIa, respectively, were eluted. Similar findings have been described by other investigators (13, 14).

We earlier noted that Tab failed to block platelet aggregation induced by ADP or thrombin (9). Rabbit antibodies directed against purified, denatured IIb or IIIa also had no effect on platelet aggregation or on fibrinogen binding (15, 16). In contrast, a human alloantibody directed against glycoprotein IIb-IIIa blocked both aggregation and fibrinogen binding (17, 18, 39). These data suggest that the localization of a specific antigenic determinant on the IIb-IIIa complex may help define the critical region for fibrinogen binding and platelet aggregation.

We have applied the CIE technique to localize the site of Tab binding to the IIb polypeptide and also to localize the binding of a newly described monoclonal antibody, $T_10$, to a determinant created only by the association of IIb and IIIa. Studies with these antibodies suggest that the association of IIb and IIIa in the platelet membrane is required, but not sufficient, for fibrinogen binding and platelet aggregation to occur.

**EXPERIMENTAL PROCEDURES**

Hirudin, diisopropylphosphofluoridate, prostaglandin $E_2$, Sepharose CL-2B, Sephadex G-25, Type I agarose ($M_r = 0.10-0.15$), Triton X-100

*The abbreviations used are: CIE, crossed immunoelectrophoresis; SDS, sodium dodecyl sulfate; PGE$_2$, prostaglandin $E_2$; PHP, platelet-rich plasma.
Platelet Membrane Glycoprotein IIb-IIIa

X-100, Lubrol PX, ADP, and epinephrine were obtained from Sigma. Carrier-free Na221 was from Amersham. Monoclonal anti-HLA antibody and goat IgG directed against mouse IgG were purchased from Bethesda Research Laboratories. DEAE-cellulose (DE52) was obtained from Whatman. A建议on A oil was from J. G. Biddle Co. (Plymouth Meeting, PA). All other chemicals were reagent grade.

Platelet membranes were isolated by differential centrifugation from blood anticoagulated with acid/citrate dextrose and PGE1, as described by George et al. (19). For platelet aggregation and binding studies, blood obtained from aspirin-free, normal donors was anticoagulated with a 1:10 volume of 3.8% sodium citrate. The blood was centrifuged at 180 g for 10 min and 8-10 ml of platelet-rich plasma were applied to a 50-ml Sepharose CL-2B column in a plastic syringe. The platelets were eluted with Tyrode's buffer (0.138 M NaCl, 0.029 M KCl, 0.012 M NaHCO3, 0.036 M Na2HPO4/H2O, pH 7.4) that contained 3.5 mg/ml of bovine serum albumin and 5.5 mM glucose. The platelet count of the pooled fractions was determined with a Trithemocounter (Couler Electronics). Fibrinogen binding experiments and platelet aggregation studies were performed within 1 h after gel filtration.

Human mononuclear cells were prepared from defibrinated blood by centrifugation over ficoll-Hyphaque (20) and washed three times in phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH2PO4, 3.17 mM Na2HPO4, pH 7.4) before use. The T-lymphoblastoid cell line SCRF-CEM (21) was a gift from Dr. Parker Suttle (San Antonio).

Monoclonal Antibodies—Hybridomas were generated from a cell fusion performed previously and described in detail (9). The Tab antibody is derived from clone 1 of the 24 hybridomas producing anti-platelet antibodies (9). The antibodies produced by the remaining hybridomas were tested for platelet specificity with a solid phase radioimmunoassay essentially as described by Stocker and Heusser (22). 10^10 platelets were tested for platelet specificity with a solid phase radioimmunoassay except where other methods are specifically stated. Direct binding of 125I-monoclonal IgG was performed as described previously (9), except that reaction mixtures were in Tyrode's buffer, pH 7.4, containing 3.5 mg/ml of bovine serum albumin and 5.5 mM glucose. In some experiments, binding of antibody was measured directly in platelet-rich plasma obtained as rapidly as possible after venipuncture in the presence of inhibitors added to prevent thrombin generation and platelet activation. Blood was collected directly into a syringe containing final concentrations of 0.38% sodium citrate, 2 units/ml of hirudin, 1 μg/ml of PGE1, 1 mM dibutyryl CAMP, and 2 mM diisopropylphosphorylfluoridate. Platelet-rich plasma was immediately prepared by centrifuging blood at 25,000 × g at 12,000 × g in an Eppendorf microfuge. Aliquots of PRP were then added to Tyrode's buffer containing the above inhibitors and incubated with 125I-monoclonal IgG for 7 min or 30 min at room temperature. After centrifugation over a 9:1 mixture of n-butyl phthalate and Apiezon A oil (9), the platelet pellets were counted. The experiments were completed within 15 min and 40 min, respectively, after venipuncture. The platelets remained unreactive as assessed by the inability of PRP to aggregate in response to 20 μM ADP, 20 μM epinephrine, and 1 unit/ml of thrombin.

Fibrinogen binding was performed as described by Bennett and Vilase (1), with slight modifications. Suspensions of 5 × 10^5 gel-filtered platelets were mixed with 1 mM CaCl2 and labeled fibrinogen in 0.038 M sodium citrate, 0.036 M Na2HPO4, pH 7.4, of 1.25 × 10^5 gel-filtered platelets in Tyrode's buffer containing 3.5 mg/ml of bovine serum albumin, 5 mM glucose, 1 mM CaCl2, and 200 μg/ml of fibrinogen. Aggregation was initiated by adding 10 μM ADP or epinephrine. Thromboxane B2 was determined in parallel tubes containing 20-fold excess of unlabeled fibrinogen or with tubes in which ADP was omitted; identical results were obtained by either method. Non-specific binding was subtracted from total binding to determine specific binding. In some experiments, unlabeled monoclonal IgG or Fab fragments were incubated with platelets and labeled fibrinogen before addition of ADP.

Platelet Aggregation—Platelet aggregation was measured at 37°C in an uncoated glass cuvette at a constant stirring rate of 1200 rpm. Platelet suspensions of 0.5 ml consisted either of citrated platelet-rich plasma or of 1.25 × 10^5 gel-filtered platelets in Tyrode's buffer containing 3.5 mg/ml of bovine serum albumin, 5 mM glucose, 1 mM CaCl2, and 200 μg/ml of fibrinogen. Aggregation was initiated by adding 10 μM ADP or epinephrine. Thromboxane B2 content was determined in parallel tubes containing 20-fold excess of unlabeled fibrinogen. In some experiments, monoclonal IgG or Fab fragments were incubated with platelets for 15–30 min before addition of the agonist. Changes in light transmission were recorded with a Payton dual-channel aggregometer.

Other Methods—The protein concentration of solubilized platelets was determined according to Markwell et al. (28). The protein concentration of mouse IgG and Fab fragments were estimated from absorbance at 280 nm using an estimated E280 of 15 and 14, respectively (29). Fibrinogen protein concentration was determined assuming a E280 of 15.1 (30). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (11). Reduced samples contained 5% mercaptoethanol. Radioactive iodide was measured in a Tracor Model 1191 γ counter.

RESULTS

Identification of the Monoclonal Anti-platelet Antibody T10—On the basis of the screening radioimmunoassay as described under "Experimental Procedures," we selected hybridoma cells which were producing IgG antibodies apparently specific for platelets. These cells were cloned and rescreened and then monoclonal IgG was purified to homogeneity as determined by SDS-polyacrylamide gel electrophoresis. One

protein were electrophoresed at 10 V/cm at 12.5°C for 60 min in a first dimension gel consisting of 1% agarose and 1% Triton X-100 in the Tris/glycine buffer. Second dimension electrophoresis was performed at 0.5 V/cm for 18 h at 12.5°C against an intermediate gel and then an upper gel, both containing 1% agarose and 1% Triton X-100 in Tris/glycine buffer. The intermediate gel contained 100 μg (1 μg/cm2) of 125I-monoclonal IgG. Polyclonal rabbit anti-platelet IgG (0.6 mg/cm2) was incorporated in the upper gel. In some experiments, 1% Lubrol PX was used instead of Triton X-100 with identical results. After electrophoresis, the gels were washed, dried, and stained with Coomassie blue R (27). Autoradiography was performed by exposing the dried gel to Kodak AR film in a cassette containing a DuPont Cronex Lightning Plus intensifying screen.
of these clones, designated T10, was selected for detailed studies. T10 IgG was coupled to Protein A-Sepharose and Triton X-100-solubilized platelets were applied to the column, as described in our previous studies of the Tab antibody (10). Bound protein eluted with 3% SDS and analyzed on SDS gels revealed two bands with the characteristic mobilities (9, 10) of platelet membrane glycoproteins IIb and IIIa and a faint band co-migrating with platelet actin (not shown). Thus, the T10 antibody, like Tab, recognizes a determinant on the glycoprotein IIb-IIIa complex.

Localization of Antigenic Determinants—We employed the technique of crossed immunoelectrophoresis as developed by Hagen et al. (11) and Kunicki et al. (12) to identify the areas on glycoprotein IIb-IIIa to which the Tab and T10 antibodies bind. Figs. 1 and 2 illustrate typical CIE patterns obtained when solubilized platelets were electrophoresed against polyclonal rabbit anti-platelet antibodies. 125I-Tab was incorporated in the intermediate gels seen in Fig. 1 and 125I-T10 in the intermediate gels seen in Fig. 2. The Coomassie blue-stained patterns were identical in both cases. The predominant immunoprecipitate seen in Figs. 1A and 2A represents the glycoprotein IIb-IIIa complex (11, 12). When platelets were solubilized in the presence of 5 mM EDTA (Figs. 1B and 2B), most of the IIb-IIIa complex dissociated into distinct immunoprecipitates representing IIb and IIIa, respectively (12). Autoradiograms of the gels (Figs. 1C and 2C) indicated that both 125I-Tab and 125I-T10 bound to the IIb-IIIa complex, as predicted from our affinity chromatography experiments. After platelet solubilization in EDTA, 125I-Tab bound not only to the residual IIb-IIIa complex, but also to the arc representing dissociated IIb (Fig. 1D). In contrast (Fig. 2D), 125I-T10 bound only to the undissociated IIb-IIIa complex. No binding of 125I-T10 to either dissociated IIb or IIIa was seen even after prolonged exposure of the film. Identical results were obtained when CIE was performed using the previously characterized rabbit anti-platelet antibodies used by Kunicki et al. (12) and given to us for these experiments (data not shown). Thus, the Tab antibody recognizes a determinant on IIb, while T10 recognizes a determinant formed only after the calcium-mediated association of IIb and IIIa into the intact complex.

Binding of T10 and Tab to Platelets—We next performed direct binding studies with 125I-T10 IgG to determine the number of T10 binding sites on platelets. We used gel-filtered platelets for these studies, but have obtained similar results with platelet-rich plasma (see below) and with washed platelets. In preliminary experiments, we determined that binding of T10 was complete by 30 min. Therefore, all subsequent studies were performed using a 30-min incubation with 125I-T10. Fig. 3 illustrates a representative binding experiment. 125I-T10, as in previous studies with 125I-Tab (9), bound with high affinity to a single class of binding sites on human platelets. Nonspecific binding was negligible since greater than 99.5% of bound 125I-T10 IgG was displaced when a 100-fold excess of unlabeled T10 IgG was added simultaneously. In this experiment, 125I-T10 bound to 53,000 sites/platelet with a dissociation constant of 8.5 nM.

In simultaneous experiments, gel-filtered platelets from six normal subjects bound 50,600 ± 5,600 (S.D.) 125I-T10 molecules/platelet (KD = 8.6 nM) and 47,800 ± 11,200 125I-Tab molecules/platelet (KD = 6.2 nM). These values are essentially the same as the 40,000 Tab binding sites reported previously with platelets isolated by differential centrifugation (9). The equivalent number of binding sites seen with each antibody suggests that there is one IIb polypeptide per IIb-IIIa complex and is consistent with IIb-IIIa being a heterodimer (32, 33).

**FIG. 1.** Use of crossed immunoelectrophoresis to identify the binding site of the monoclonal antibody, Tab. One hundred μg of platelet protein solubilized in 1% Triton X-100 in the absence (A, C) or presence (B, D) of 5 mM EDTA were electrophoresed from left to right in the first dimension. Electrophoresis in the second dimension was through an intermediate gel containing 10⁶ cpm (0.1 μg/cm²) of 125I-Tab, then through an upper gel containing 0.6 mg/cm² of polyclonal rabbit anti-human platelet antibody. The gels were washed, dried, stained with Coomassie blue R, then exposed to Kodak X-Omat AR film for 1 h. A and B represent the Coomassie blue-stained gels, C and D the corresponding autoradiographs.

**FIG. 2.** Use of crossed immunoelectrophoresis to identify the binding site of the monoclonal antibody, T10. Experimental conditions were identical with those in Fig. 1, except that 10⁶ cpm (0.1 μg/cm²) of 125I-T10 were incorporated in the intermediate gel.
The binding sites for T10 and Tab do not appear to be adjacent to each other, since a 100-fold excess of either unlabeled antibody does not inhibit binding of the other radiolabeled protein (not shown). Binding of both T10 and Tab was the same in unstimulated platelets and platelets treated with 10 μM ADP. However, only platelets stimulated with ADP were able to aggregate or bind 125I-fibrinogen (see below).

The binding of 125I-Tab to platelets was unaffected by the addition of EDTA or CaCl2. In contrast, addition of 5 mM EDTA to platelets reduced 125I-T10 binding by approximately 60% (Table I). T10 binding could be partially restored by addition of 10 mM CaCl2 to the EDTA-treated platelets. We were unable to reduce binding of T10 below 40% of control values, even with platelets isolated in the continuous presence of 5 mM EDTA, as well as the metabolic inhibitors 0.1 M dibutyryl cAMP and 1 μg/ml of PGE2. The same degree of inhibition was seen in Tyrode's buffer at pH 8.5 or at pH 7.4.

We considered the possibility that T10 might bind to platelets because of the association of IIb and IIIa in the membrane during the platelet isolation procedure. To examine this question, we rapidly prepared platelet-rich plasma in the presence of inhibitors designed to minimize platelet activation, then immediately measured T10 and Tab binding (see "Experimental Procedures"). Under these conditions, binding of T10 and Tab was not different (Table II) and was comparable to that seen with washed platelets at the same antibody concentrations and incubation times. This suggests that the isolation procedure itself did not cause association of IIb and IIIa.

Inhibition of Fibrinogen Binding by T10—To see if Tab and T10 might identify domains of functional significance in glycoprotein IIb-IIIa, we investigated the effects of both antibodies on fibrinogen binding to platelets. We found that 125I-fibrinogen bound to a single class of binding sites with approximately 45,000 molecules bound per platelet at saturation, confirming the data of other investigators (1-3). Fibrinogen binding was calcium-dependent and required platelet stimulation with an agonist such as ADP. When platelets were preincubated with T10 IgG, 125I-fibrinogen binding was reduced to approximately 35% of control values (Fig. 4). T10 Fab fragments also inhibited fibrinogen binding but, in some experiments, to a lesser extent than the intact IgG molecule. The less effective inhibition of fibrinogen binding by the Fab fragments was not due to alteration of Fab specificity by papain digestion, since the inhibition of 125I-fibrinogen binding to platelets was the same with unlabeled intact T10 or T10 Fab fragments.

In contrast to T10, Tab or nonimmune mouse IgG or Fab fragments either had no effect on fibrinogen binding or slightly augmented binding (Table III). The mechanisms for augmentation of binding were not investigated, but similar findings have been reported for nonimmune human IgG (18) and rabbit F(ab')2 fragments directed against IIb (16).

**Table I**

| IgG bound          | Tab | T10 |
|--------------------|-----|-----|
| Control            | 613 | 693 |
| EDTA               | 582 | 277 |
| EDTA + CaCl2       | 641 | 499 |

**Table II**

**Binding of Tab and T10 in platelet-rich plasma**

PRP was rapidly prepared by centrifuging citrated blood containing 2 units/ml of hirudin, 2 mM diisopropylphosphofluoridate, 1 mM dibutyryl cAMP, and 1 μg/ml of PGE2; at 12,000 X g for 2.5 s. Aliquots of PRP were then incubated with 2 μg/ml of 125I-Tab or 125I-T10 IgG for 7 min or 30 min and then centrifuged and bound radioactivity was measured. Results are means of duplicate determinations and are representative of two similar experiments. The range of the duplicates was within 10% of the mean.

| Incubation time | Tab | T10 |
|-----------------|-----|-----|
| 7 min           | 129 | 129 |
| 30 min          | 615 | 561 |

**Fig. 4. Inhibition of 125I-fibrinogen binding to platelets by the monoclonal antibody, T10. Platelets, 107/ml in Tyrode's buffer, pH 7.4, containing 3.5 mg/ml of bovine serum albumin and 1 mg/ml of glucose, were incubated for 30 min in the presence or absence of 5 mM EDTA. Calcium chloride at a final concentration of 10 mM was then added to half of the tubes containing EDTA. After an additional 30 min, 125I-Tab or 125I-T10 IgG, 4 μg/ml final concentration, was added. After an additional 30 min, the platelets were centrifuged and bound 125I-IgG was measured. Results are means of duplicate determinations. The range of the duplicates was within 10% of the mean.**

**Fig. 3. Steady state binding of 125I-T10 to human platelets.** Reaction mixtures contained 107 platelets/ml and varying amounts of 125I-T10 in Tyrode's buffer, pH 7.4, containing 3.5 mg/ml of bovine serum albumin and 1 mg/ml of glucose. After a 30-min incubation at room temperature, bound radioactivity was measured as described under "Experimental Procedures." The inset represents a Scatchard plot (44) of the binding data.
Effects of antibodies on fibrinogen binding to platelets

Tab and T10 bound to the immunoprecipitate representing the intact IIb-IIIa complex. After dissociating IIb and IIIa with EDTA, we identified specific binding of 125I-Tab only to IIb. In contrast, 125I-T10 failed to bind to either IIb or IIIa separately. Thus, T10 recognizes a determinant formed only after the association of IIb and IIIa, most likely a region where the two polypeptides interact. Alternatively, the association of the two polypeptides creates a new antigenic determinant on IIb or IIIa remote from the actual regions of contact. One of the advantages of the CIE system is that antigenic determinants are analyzed under nondenaturing conditions. This may be of particular importance in examining the binding of monoclonal antibodies with restricted specificity to secondary or tertiary structures. We were unsuccessful in identifying the site of binding of Tab by “Western blot” techniques (34, 35) in which 125I-Tab was overlaid over platelet proteins separated on SDS-gels and transferred to activated paper (not shown), presumably because of denaturation of the antigenic determinant on IIb. Therefore, a negative result with the blotting procedure would not have distinguished the antigenic specificities of Tab and T10.

Recent analyses of Triton X-100-solubilized glycoprotein IIb-IIIa by gel filtration and sucrose gradient ultracentrifugation suggest that the IIb-IIIa complex is a heterodimer (32, 33). The equivalent number of platelet binding sites that we find for both 125I-Tab and 125I-T10 is consistent with the data of these investigators. It is notable that neither antibody inhibits binding of the other, which indicates that the externally oriented portion of IIb-IIIa encompasses enough area to allow simultaneous binding of at least two intact IgG molecules.

The reversible inhibition of 125I-T10 binding in intact platelets by EDTA indicates that the calcium-mediated association of IIb and IIIa occurs in the membrane itself and is not a phenomenon occurring only after solubilization in nonionic detergents. We were unable to inhibit T10 binding completely with EDTA. This is probably due to inaccessibility to the chelator of a portion of the calcium ions bound to IIb-IIIa within the membrane. Investigators using CIE have also pointed out that complete dissociation of IIb and IIIa may require prolonged incubation with EDTA and may be more effective at alkaline pH (12, 36). We noted no difference in 125I-T10 binding in Tyrode’s buffer containing 5 mM EDTA at pH 8.5 or pH 7.4, although it is conceivable that local pH within the membrane was not affected by the change in buffers. We did not incubate platelets with EDTA longer than 2 h. The reasons for incomplete restoration of T10 binding with added CaCl₂ are also not clear. Jennings and Phillips (32), using purified IIb and IIIa in solution in Triton X-100, also noted that reformation of the complex was incomplete after addition of CaCl₂.

We originally hypothesized that IIb and IIIa are polypeptide subunits of a single membrane glycoprotein (9, 10). After platelet stimulation, some additional change, perhaps a slight conformational shift in one or both of the subunits, would allow fibrinogen to bind to the glycoprotein. An alternative hypothesis is that IIb and IIIa exist as discrete glycoproteins in the resting platelet, but associate together after platelet activation, perhaps as a result of shifts in membrane calcium. In this view, solubilization of platelets in nonionic detergent would promote complex formation of IIb and IIIa because of small amounts of calcium made available during solubilization. Polley et al. (37) used immunoelectron microscopy with anti-IIb and anti-IIIa antibodies to demonstrate the clustering of IIb and IIIa in platelet membranes after thrombin activation. However, the resolution obtained with their electron micrographs would not distinguish association of IIb and IIIa

Table III

| Antibody Type          | Fibrinogen Specifically Bound (molecules × 10⁻¹⁷/platelet) |
|------------------------|------------------------------------------------------------|
| Control                | 49                                                         |
| T10 IgG                | 20                                                         |
| T10 Fab                | 21                                                         |
| Tab IgG                | 52                                                         |
| Tab Fab                | 55                                                         |
| Nonimmune mouse IgG    | 58                                                         |
| Nonimmune mouse Fab    | 41                                                         |

Fig. 5. Effect of antibodies on ADP-induced platelet aggregation. Citrated platelet-rich plasma was preincubated for 15 min at 37 °C with 100 μg/ml of Tab IgG or 20 μg/ml of T10 IgG. ADP, 10 μM final concentration, was then added and changes in light transmission followed. The control tracing represents superimposable curves obtained when PRP was preincubated with no antibody or with 20 μg/ml of nonimmune mouse IgG or 100 μg/ml of nonimmune Fab fragments.

seen with washed platelets. Tab IgG either had no effect, or, as seen in Fig. 5, slight inhibition of the rate and total extent of platelet aggregation. Tab Fab fragments and nonimmune mouse IgG or Fab fragments had no effect. In contrast, T10 IgG completely blocked aggregation except for a small initial wave of reversible aggregation. T10 Fab fragments caused a modest, but less consistent inhibition of aggregation. In general, thrombin-induced aggregation of platelets was inhibited in similar fashion, although in one experiment aggregation reached nearly normal levels after a delay of 8–10 min (not shown). The oscillating baseline tracing seen before the addition of ADP is characteristic of unstimulated discoid platelets which randomly interfere with light transmission as they are stirred. After activation, platelets undergo a shape change becoming spherical cells with small pseudopods. The oscillating pattern on the tracing is no longer seen, because the spherical platelets now uniformly affect light transmission during stirring. T10 IgG did not affect shape change, as seen by the normal flattening of the aggregometer tracing after the addition of ADP.

**DISCUSSION**

In this study, we have employed crossed immunoelectrophoresis to localize the antigenic determinants of two monoclonal antibodies to glycoprotein IIb-IIIa. As expected, both

**TABLE III**

| Antibody Type          | Fibrinogen Specifically Bound (molecules × 10⁻¹⁷/platelet) |
|------------------------|------------------------------------------------------------|
| Control                | 49                                                         |
| T10 IgG                | 20                                                         |
| T10 Fab                | 21                                                         |
| Tab IgG                | 52                                                         |
| Tab Fab                | 55                                                         |
| Nonimmune mouse IgG    | 58                                                         |
| Nonimmune mouse Fab    | 41                                                         |
polypeptides into heterodimers or the clustering of pre-existing heterodimers into localized areas of the membrane. Since identical numbers of T10 binding sites are seen in unstimulated platelets and platelets stimulated with ADP, we conclude that platelet activation is not required for association of IIb and IIIa in the membrane. Our "unstimulated" platelets were not activated prematurely by gel filtration, since they did not bind fibrinogen or aggregate until ADP was added. Furthermore, T10 and Tab binding were identical in platelet-rich plasma obtained rapidly after venipuncture in the presence of several inhibitors of thrombin generation and platelet activation. Finally, we have obtained similar binding data using 125I-Fab fragments (not shown), indicating that membrane association of IIb and IIIa was not produced by cross-linking effects from divalent IgG. Our data therefore suggest that IIb and IIIa are associated in circulating unstimulated platelets and thus can be accurately defined as subunits of a single membrane glycoprotein. Glycoprotein IIb-IIIa is analogous to proteins such as hemoglobin and the acetylcholine receptor which consist of distinct polypeptide subunits associated by noncovalent interactions. It is not yet known if IIb and IIIa are derived from common or separate genes, although structural studies of the peptides suggest that the latter possibility is more likely (9, 15).

There is considerable evidence supporting the role of glycoprotein IIb-IIIa in the binding of fibrinogen to platelets, but it has been unclear as to whether one or both polypeptide subunits are required. Using an enzyme-linked immunosorbent assay, Nachman and Leung (16) demonstrated calcium-dependent binding of fibrinogen to a partially purified preparation of IIb-IIIa. However, they were unable to prepare separated IIb and IIIa without protein denaturation and could not determine whether one or both polypeptides were required for fibrinogen interaction. Platelet binding studies with fibrinogen modified with a photoreactive cross-linker provided evidence for specific binding of fibrinogen to IIb-IIIa, but did not rule out an interaction with IIb undetected by the cross-linker (38). It is significant that non-cross-reacting rabbit antibodies to IIb and to IIIa, prepared by immunization with the individual polypeptides, failed to inhibit fibrinogen binding or platelet aggregation (15, 16). This is consistent with the lack of inhibition of these functions by the anti-IIb monoclonal antibody, Tab. Our finding that only T10 inhibits platelet fibrinogen binding suggests that IIb and IIIa must interact in order for fibrinogen binding to occur. Our results are supported by experiments with a human alloantibody to glycoprotein IIb-IIIa which inhibits fibrinogen binding and platelet aggregation (17, 18). When analyzed by CIE, this antibody also appears to bind to a determinant created only by the association of IIb and IIIa (39). Preliminary reports have appeared of other monoclonal antibodies to glycoprotein IIb-IIIa that inhibit fibrinogen binding, but the specific antigenic determinants were not identified (40, 41). Recently, Gogstad et al. (42) demonstrated calcium-dependent binding of 125I-fibrinogen to the IIb-IIIa immunoprecipitate in CIE gels. Immunoprecipitates of dissociated IIb and IIIa did not react with fibrinogen. These results also suggest a requirement for association of IIb and IIIa in order for fibrinogen binding to occur. It is likely that fibrinogen binds to a domain where IIb and IIIa are in close proximity, although the association of the subunits might cause a conformational change in another region of either IIb or IIIa where fibrinogen would then interact.

It appears that T10 binds to a region near to, but not precisely corresponding to, the fibrinogen binding site of IIb-IIIa, since fibrinogen binding is not totally inhibited by T10 IgG and even less impaired by Fab fragments. Incomplete inhibition may also be explained by the observation of Mar-guerie et al. (43) that fibrinogen becomes irreversibly bound to platelets with increasing time of incubation. If a similar phenomenon occurred in our system, some bound fibrinogen molecules might become resistant to competitive displacement by T10.

In summary, our results suggest that IIb and IIIa exist as polypeptide subunits of a single membrane glycoprotein in unstimulated platelets. Fibrinogen appears to bind to the glycoprotein at or near a region created by the interaction of the two subunits. Platelet activation with an agonist such as ADP is not required for association of IIb and IIIa but is necessary to promote fibrinogen binding and platelet aggregation. Therefore, some additional event in addition to membrane association of IIb and IIIa must be required for fibrinogen binding to proceed. Possible events include a conformational shift in glycoprotein IIb-IIIa, clustering of IIb-IIIa heterodimers thereby increasing their binding affinity for fibrinogen (37), or interaction of other unknown membrane components with the glycoprotein.

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