Monoclonal Antibodies Bound to Subunits of the Integrin GPIIb-IIIa Are Internalized and Interfere With Filopodia Formation and Platelet Aggregation

By William M. Isenberg, Dorothy F. Bainton, and Peter J. Newman

The monoclonal antibodies Tab and AP3 are directed, respectively, against GPIIb and GPIIIa, the subunits of the platelet fibrinogen receptor. When added together to platelets, these antibodies prevent adenosine diphosphate (ADP)-induced platelet aggregation, despite normal fibrinogen binding (Newman et al, Blood 69:686, 1987). To explore the cellular requirements of aggregation after fibrinogen binding, we used several techniques to study platelets treated with Tab and AP3, then stimulated with ADP. We used scanning and transmission electron microscopy to evaluate platelet morphology, immunolabel-surface replication to determine whether individual GPIIb-IIIa complexes clustered, immunocytochemistry on frozen thin sections to study the subcellular distribution of the integrin GPIIb-IIIa and fibrinogen, and biochemical methods to assess the activation of the platelet cytoskeleton. We found that the treated cells had short, blunted projections instead of normal filopodia. Other morphologic abnormalities, apparent in thin section, were aberrantly placed α-granules and microtubules, and a prominent, worm-like, fibrinogen-filled surface-connected canalicular system. Biochemical analysis suggested that such platelets undergo massive actomyosin-controlled membrane flow, which serves to sequester GPIIb-IIIa and makes the platelets refractory to aggregation. We conclude that aggregation requires the formation of long, slender filopodia, probably directed by cytoskeletal rearrangements after activation, and that the transmembrane GPIIb-IIIa complex may play a role in signaling these events.

© 1990 by The American Society of Hematology.

MATERIALS AND METHODS

Platelet aggregation. Platelet aggregation was monitored with an aggregometer (Payton Bio/Data Platelet Aggregation Profiler, model PAP-2A (Bio/Data Corp, Willow Grove, PA), to determine the dosages of Tab and AP3 required for the synergistic inhibition of ADP-induced platelet aggregation. The concentrations were 20 μg/mL for both antibodies. Aggregometry was performed in parallel with electron microscopic studies to ensure functionality of the antibodies on each day.

Surface replication. Platelets from each group were processed for the immunolabel-surface replica technique described previously. The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1990 by The American Society of Hematology.

From the Department of Pathology, University of California San Francisco, CA; and the Blood Center of Southeastern Wisconsin, Milwaukee, WI.

Submitted November 17, 1989; accepted June 18, 1990.

Supported by Grant No. HL-31610 from the National Institutes of Health (to D.F.B.) and Grant-in-Aid No. 85-730 (to P.J.N.) from the American Heart Association. W.M.I. is a recipient of a Regents Scholarship from the University of California.

Address reprint requests to William M. Isenberg, PhD, Department of Pathology, Box 0506, University of California, Third and Parnassus Ave, San Francisco, CA 94143-0506.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.
parafomaldehyde and 0.05% glutaraldehyde in phosphate buffer, pH 7.2, for 1 hour at 4°C, and were then rinsed in phosphate-buffered saline + 50 mmol/L NH₄Cl to quench excess aldehyde groups in the fixative. Fixed platelets were settled onto poly-L-lysine-coated coverslips, postfixed in 1% aqueous osmium tetroxide, dehydrated in graded ethanol at 4°C, and critical point-dried using carbon dioxide as a transition fluid. Critical point-dried specimens were sputter-coated with platinum-carbon/carbon electrodes and examined with a JEOL 100CXII scanning/transmission electron microscope at an accelerating voltage of 20 kV.

Movement of antigen-antibody complexes. To monitor the movement of the Tab- and AP3-labeled fibrinogen receptor throughout the platelet activation process, we pretreated washed platelets with the following antibody combinations: Tab alone, AP3 alone, or Tab and AP3 together. The cells were exposed to the antibodies for 5 minutes at 25°C, either with or without added fibrinogen (0.25 mg/mL). After this time, protein A coupled to 5-nm colloidal gold (Janssen Pharmaceuticals, Beerse, Belgium) was added, and the cells were either kept at 25°C or warmed to 37°C. After 5 minutes of incubation with protein A-gold, the platelets were either fixed immediately or stimulated with 20 mmol/L ADP for 5 minutes at 37°C and then fixed. The fixation protocols used either fixation for 4 hours at 4°C in the 4% paraformaldehyde-lysine-periodate fixative of McLean and Nakane, followed by embedding in 2.3 mol/L sucrose for subsequent frozen thin sectioning, or fixation in the 4% glutaraldehyde and 40 mmol/L lysine fixative of Boyles et al. With the latter fixative, no protein A-gold was used as a marker, and platelets were fixed for 30 minutes at 4°C, rinsed in Michaelis’ acetone veronal buffer, postfixed in 1% OsO₄ for 15 minutes, and rinsed in distilled H₂O. Following an overnight en bloc staining in 1% uranyl acetate, the cells were dehydrated, infiltrated with the resin, and embedded in the same resin. Thin sections were examined using a double labeling of both the integrin (5 nm) and the ligand fibrinogen (10 nm).

Frozen thin-sectioning immunocytochemistry. Fixed cells, embedded in sucrose as described above, were frozen thin sectioned on a Reichert Ultracut E microtome. The sections were exposed to polyclonal rabbit anti-human fibrinogen, followed by protein A-gold 10 nm in diameter. Because the tissue had been exposed to Tab and AP3 and protein A-gold 5 nm before sucrose embedding, the resulting images gave us a double labeling of both the integrin (5 nm) and the ligand fibrinogen (10 nm).

Biochemistry. Cytoskeletons from cells that had undergone lactoperoxidase-catalyzed radiiodination of surface glycoproteins were isolated using the procedures of Fox. Iodination was performed as described by Phillips and Agin. Briefly, antibody-treated, lactoperoxidase-labeled platelets were lysed by the addition of an equal volume of an ice-cold Tris lysis buffer containing 2% Triton X-100, with 2 mg leupeptin/mL, 100 mmol/L benzamidine, and 2 mmol/L phenylmethylsulfonylfluoride added as inhibitors. Following lysis, the material was microfuged for 4 minutes, and the resulting supernatants were ultracentrifuged at 100,000g for 2.5 hours. The cytoskeletal pellets derived from this procedure were solubilized in sodium dodecyl sulfate by the method of Laemmli. Samples were loaded onto 7.5% polyacrylamide gels with a 3% stacking gel and electrophoresed overnight at 25 V. The resolving gels were then stained in Coomassie Brilliant Blue R. Dried gels were exposed to Kodak X-5 x-ray film and autoradiograms were prepared to determine whether the [125I]-labeled GPIIb-IIIa IIIa had associated with the actin cytoskeletons. As a positive control for this association, some platelets were stimulated with thrombin and allowed to aggregate for 15 minutes, conditions under which it has been shown that the GPIIb-IIIa complex associates with actin. These thrombin-activated, aggregated platelets were processed along with the antibody-treated cells.

RESULTS

Effect of monoclonal antibodies AP3 and Tab on platelet aggregation. As has been previously shown, platelets aggregated normally when they were suspended in plasma, treated with either Tab alone or AP3 alone, and then stimulated with ADP (Fig 1A). Aggregometry tracings of these platelets were neither quantitatively nor qualitatively different from the tracings of untreated, ADP-stimulated platelets. However, when platelets were treated with Tab and AP3 together before ADP stimulation, they failed to aggregate (Fig 1A). Earlier studies have shown that fibrinogen binding is quantitatively normal in all three of these experimental groups.

Effect of Tab and AP3 on filopodia formation and platelet shape. To study the effects of Tab and AP3 on platelet morphology, we used scanning electron microscopy. Untreated, resting platelets looked discoid by this technique (Fig 1B). Platelets that were not treated (Fig 1C), or were treated with either Tab (Fig 1D) or AP3, before ADP stimulation, developed long, slender filopodia and aggregated. However, platelets treated with both Tab and AP3 before ADP stimulation developed only blunted, foreshortened projections (Fig 1E) and did not aggregate.

Fibrinogen receptor clustering. Examination of surface replicas revealed GPIIb-IIIa clustering in all ADP-stimulated platelets, whether pretreated with Tab, AP3, or both. We illustrate the combined Tab and AP3 pretreatment group (Fig 1F), although clustering was qualitatively and quantitatively similar in all groups. This finding is consistent with our earlier observation that GPIIb-IIIa clustering is dependent on fibrinogen binding, which is not affected by any of the antibody pretreatments.

Examination of the surface replicas confirmed that platelets treated with Tab and AP3 lacked the typical long, slender filopodial projections present in all of the other ADP-stimulated groups.

Thin-section morphology: transmission electron microscopy and immunocytochemistry. Transmission electron microscopy provided additional information about morphologic changes in platelets treated with Tab and AP3. Figure 2A shows untreated, ADP-stimulated platelets. The α-granules have centralized within the microtubular coil, resulting in an organelle-poor zone at the periphery, and the cells have structurally normal filopodia. The cells aggregated with one another, their filopodia forming complex interdigitations. Treatment with Tab and AP3 before ADP stimulation resulted in striking morphologic alterations. First, in approximately one third of the cells, the surface-connected canalicular system (SCCS) was prominent, tortuously worm-like, and filled with an electron-dense product (Fig 2B). Well-formed aggregates were not present. When cells abutted each other, it appeared to be more an artifact of centrifugation than a true interaction of filopodia (Fig 2B). Microfilaments, consistent with actin, could be seen condensed around the canalicular membranes in these platelets. We hypothesized that the antibody treatment had activated membrane flow, and that the fibrinogen receptors had moved into the vermiform SCCS. To verify this impression, we analyzed the movement of Tab and AP3-tagged fibrinogen receptors by incubating the platelets with protein A-gold, at either 25°C...
Fig 1. (A) Aggregometry tracings of platelets stimulated with ADP after treatment with Tab, AP3, or both. Only Tab and AP3 in combination inhibited aggregation. %T, percent transmittance. Scanning electron microscopy of (B) resting platelets, (C) platelets stimulated with ADP, (D) platelets treated with Tab before ADP stimulation, and (E) platelets treated with both Tab and AP3 before ADP stimulation. (F) Surface replica of platelets pretreated with both Tab and AP3 before ADP stimulation. Clustering of the immunoprobe was seen in all ADP-stimulated groups, regardless of pretreatment. (B through D: magnification ×18,000; E: magnification ×55,000.)
or 37°C. In platelets incubated with the gold label at 25°C, the fibrinogen receptors moved to the forming invaginations of the plasma membrane (Fig 3A) that lay between the blunted, foreshortened projections observed by scanning electron microscopy. In platelets incubated at 37°C, the gold label was observed within the extensive vermiform SCCS, while there was only sparse labeling of the plasma membrane (Fig 3B). With immunocytochemistry performed on frozen thin sections, we confirmed the sequestration of GPIIb-IIIa in the SCCS, as well as the localization of fibrinogen at this site (Fig 3C). Again, the plasma membrane bore little immunoreactive GPIIb-IIIa.

Two other morphologic aberrations were seen in platelets treated with Tab and AP3 before ADP stimulation (Fig 4A). Instead of long, slender filopodial projections, there were short, cytoplasmic projections containing organelles, including α-granules, and the microtubular coil was in disarray (compare with Fig 2A). These aberrations in the microtubular coil and the filopodia prompted us to examine the cytoskeletons of platelets treated with Tab and AP3.

Cytoskeletal changes. To test the hypothesis that pre-treatment of platelets with Tab and AP3 was causing a physical association of GPIIb-IIIa with the platelet actin-based cytoskeleton, we examined the isolated cytoskeletons
Fig 3. (A and B). Transmission electron micrographs of platelets pretreated with Tpa and AP3 and protein A-gold and kept at 25°C (A) or at 37°C (B) before fixation. Immunolabeled GPIIb-IIIa (arrowheads) is seen lining plasma membrane invaginations in panel A and within the veriform SCCS in panel B. (A and B: magnifications ×60,000.) (C) Frozen thin section of a platelet prepared as in panel B, but with anti-fibrinogen antibody and a 10-nm gold label applied after thin sectioning. The 5-nm gold label for GPIIb-IIIa and the 10-nm gold label for fibrinogen are both present within the SCCS. (Magnification ×81,000.)
Fig 4. (A) Transmission electron micrograph of cells pretreated with Tab and AP3 before ADP stimulation. Cytoplasmic projections (arrowheads): α-granule (α). (Magnification ×66,000.) (B) Biochemical assessment of GPIIb-IIIa association with the actin cytoskeleton. Surface glycoproteins were labeled by lactoperoxidase-catalyzed iodination procedures (see text) and the actin cytoskeletons were isolated. The identities of the indicated membrane and cytoskeletal elements were verified by direct comparison with sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels containing known membrane and cytoskeletal components. Lane 1, whole intact platelets; lane 2, no pretreatment, no stimulation; lane 3, no pretreatment, ADP stimulation; lane 4, Tab and AP3 pretreatment, no stimulation; lane 5, Tab and AP3 pretreatment, ADP stimulation; lane 6, no pretreatment, thrombin stimulation. (C) Changes in the platelet cytoskeleton following Tab and AP3 pretreatment. Coomassie Blue-stained gel of whole platelets (lane 1) and isolated cytoskeletons (lanes 2 to 5) with a variety of treatments. Tab and AP3 pretreatment (lanes 3 and 4), even in the absence of ADP stimulation (lane 3), caused association of actin with myosin. Such an association was not seen in platelets not pretreated with antibodies (lane 2), but was seen in thrombin-stimulated cells (lane 5).
of lactoperoxidase-labeled platelets following treatment with various antibodies and agonists. In platelets stimulated with thrombin, GPIIb-IIIa complexes were associated with the cytoskeleton. However, in platelets stimulated with ADP, whether untreated or treated with Tab, AP3, or both, the GPIIb-IIIa complexes were not associated with the actin cytoskeleton (Fig 4B). When we examined the Coomassie Blue-stained gels (Fig 4C), however, it was evident that Tab and AP3 pretreatment had a profound effect on the platelet cytoskeleton itself. While there was no association of myosin with actin in untreated control platelets (lane 2), treatment with Tab and AP3 resulted in the association of myosin with platelet actin, both in unstimulated platelets (lane 3) and platelets stimulated with ADP (lane 4).

DISCUSSION

We have found that platelets treated with a combination of the monoclonal antibodies Tab and AP3 before ADP stimulation have short, blunted filopodia, instead of the long, slender projections found in untreated, ADP-stimulated platelets. Furthermore, the treated platelets have long, worm-like canalicular membrane structures, containing an ordered, electron-dense material. We hypothesize that the antibody treatment results in rapid surface-membrane flow, causing the antibody–cross-linked glycoproteins (GPIIb-IIIa) and their ligand, fibrinogen, to become sequestered within the SCCS. This “surface-cleansing” procedure, somewhat akin to receptor capping and internalization in lymphocytes, is dependent on an intact actin-myosin cytoskeleton. This explains our finding that pretreatment with Tab and AP3, even in the absence of ADP stimulation, resulted in the association of myosin with the actin cytoskeleton. In such platelets, the already "engaged" cytoskeleton cannot direct an appropriate shape change. Instead, with their foreshortened, blunted projections, often containing α-granules that have failed to centralize, these platelets are unable to support successful aggregation. The formation of long, slender filopodia is one common antecedent to platelet aggregation induced by a variety of agonists.

Behnke has reported human platelets sequentially treated with antiplatelet antibodies and cationic ferritin undergo a process of surface membrane clearing similar to that described here. However, unlike treatment with Tab and AP3, the treatment Behnke studied did not inhibit aggregation, perhaps because he used a polyclonal antibody directed against whole immunoglobulins to portions of GPIIb and/or GPIIIIa, Autoantibodies that develop in some patients, consisting of intact immunoglobulins to portions of GPIIb and/or GPIIIa, may prompt similar surface-cleansing responses in the platelet. It is unknown whether platelets bearing bound antibodies analogous to Tab and AP3 would survive in the circulation or be targeted to the spleen for removal (or sequestration). In either case, patients affected by such a process would be expected to suffer bleeding diatheses due either to the presence of platelets that are refractory to agonists or to thrombocytopenia following excessive destruction or sequestration.

ACKNOWLEDGMENT

The authors thank Dr Rodger P. McEver for his kind provision of the monoclonal antibody Tab, without which this study could not have been performed. Biochemical studies on platelet cytoskeletons were performed in the laboratories of, and with guidance from, Dr Joan E.B. Fox at the Gladstone Foundation Laboratories, San Francisco, CA. In addition, we acknowledge the excellent technical assistance of Yvonne Jacques and the considerable editorial contributions of Barbara Poetter.
REFERENCES

Monoclonal antibodies bound to subunits of the integrin GPIIb-IIIa are internalized and interfere with filopodia formation and platelet aggregation

WM Isenberg, DF Bainton and PJ Newman