Platelet–Collagen Adhesion: Evidence for Participation of Antigenically Distinct Entities

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ABSTRACT   Univalent antibody fragments prepared from a rabbit antiserum raised against whole human platelets completely inhibited adhesion of platelets to immobilized trimeric collagen in a defined, Mg^{2+}-dependent, adhesion assay. An octylglucoside extract of whole platelets completely neutralized this antibody, and all neutralizing activity bound to immobilized wheat germ agglutinin. Further fractionation on concanavalin A gave rise to subfractions that each neutralized only partially at saturation, when tested against antibody concentrations that inhibit 50% of platelet–collagen adhesion. When tested against higher antibody concentrations that completely inhibited adhesion, each subfraction had no detectable neutralizing effect, although the combined subfractions neutralized completely. This and other evidence suggests that more than one platelet entity participates in platelet–collagen adhesion. Although distinct, they appear to play interdependent roles in a single adhesion process.

Considerable evidence is accumulating that a restricted number of surface molecules are involved in cell adhesion. The most extensively studied cell adhesion molecule (CAM),[1] N-CAM (1), is a large glycoprotein originally purified from chick neural retinal cells. It apparently mediates adhesion by direct association of N-CAM molecules on adjacent cells (1). A glycoprotein has also been implicated in adhesion in the cellular slime mold *Dictyostelium discoideum*, but its role in the adhesion process is unclear (2, 3).

The approach used to identify these CAMs has been largely immunological. Rabbits were immunized with a crude membrane fraction, and univalent fragments of the immunoglobulins that were raised were used to inhibit cell–cell adhesion in an in vitro assay. In both *D. discoideum* and chick neural retina, the antibody preparation blocked adhesion completely; and, as might be expected, crude extracts of the antigenic mixture used for immunization neutralized the antibodies. In both cases, a single glycoprotein was isolated that could completely neutralize the antibody effects. This raised the possibility that in each system there might be only one crucial

[1] *Abbreviations used in this paper:* C_{bd}, extract fraction that specifically bound to a concanavalin A column; C_{ft}, extract fraction that flowed through a concanavalin A column; CAM, cell adhesion molecule; W_{bd}, extract fraction that specifically bound to a wheat germ lectin column; W_{ft}, extract fraction that flowed through a wheat germ lectin column.

CAM, although others might still exist that were not immunogenic in the injected animals.

Another experimental system under intensive investigation is the adhesion of human platelets to collagen (4, 5), which is an early step in hemostasis. This system is especially attractive in that platelets are specialized for adhesion. Platelet–collagen adhesion is also of great clinical significance in thrombotic and bleeding disorders. Early methods of quantifying this adhesion by observing the association of platelets with subendothelial collagen in blood vessels (6, 7) have been gradually supplemented by more defined assays (5, 8–11). Immunological techniques have been used in several attempts to identify molecules involved in platelet–platelet aggregation (12, 13) and adhesion (14, 15), but results in various adhesion systems have differed, in part because the assays used did not measure a well-characterized adhesion reaction.

Recently, we described a quantitative assay of platelet adhesion to purified type I trimeric collagen covalently linked to plastic coverslips (10) under well-defined experimental conditions. Adhesion in this assay required a native collagen structure and was absolutely dependent on Mg^{2+}. This assay provided an opportunity for identification of the molecular basis of this adhesion. We show here that, as expected, an antiserum raised against human platelets completely inhibited platelet–collagen adhesion in our assay, and that these effects were neutralized by platelet extracts. However, in contrast

with work with *D. discoideum* (2, 3) or neural retina (1), saturating concentrations of subfractions of the platelet extract could not completely inhibit platelet–collagen adhesion, raising the possibility that more than one entity is directly involved in adhesion in this system. In this article, we describe the results with a rabbit antiserum that led to this conclusion. In the subsequent article (16), we describe work that uses a monoclonal antibody and has identified one of the participating molecules.

MATERIALS AND METHODS

Platelet Adhesion Assay

The adhesion of gel-filtered human platelets to trimeric collagen immobilized on plastic coverslips was assayed as described previously (10). Briefly, ^{51}Cr-labeled platelets were prepared and separated from plasma proteins by gel filtration (17) in modified Tyrode's buffer, pH 7.4, containing 1 mg/ml glucose and 2 mg/ml bovine serum albumin. Type I collagen trimers, derived from lathyritic chick skin (18), were covalently coupled to plastic coverslips using a carbodiimide reagent. The coverslips were placed in Linbro wells (Flow Laboratories, Inc., McLean, VA) and covered with 0.3 ml of Tyrode's buffer containing labeled platelets. The wells were incubated for 10 min at 37°C on a gyratory shaker and washed. Adhesion, determined as ^{51}Cr bound to coverslips, reached saturation within 20 min, was Mg^{2+} dependent, and specific for collagen trimers or fibrils (10).

Preparation of Adhesion-blocking Antiserum

Rabbit antiserum was raised by immunization with whole human platelets that were prepared by gel filtration as described previously (10), except that bovine serum albumin was omitted. Platelets prepared by this method contained <0.1% plasma proteins (fibrinogen or fibronectin) by radioimmunoassay and <1% lymphocyte contamination. The rabbit was injected with 10^9 platelets in complete Freund's adjuvant and was initially boosted after 28 d with 10^8 platelets in incomplete Freund's adjuvant. Repeated boosts were made at bimonthly intervals. Sera were collected 5, 7, and 9 d after the third and subsequent boosts and pooled, and IgG was prepared by affinity chromatography on a column of protein A–agarose (Pharmacia Fine Chemicals, Piscataway, NJ). The column was equilibrated in 75 mM Na-K phosphate, 0.15 M NaCl, pH 7.2. IgG was eluted with 1 M acetic acid, neutralized with solid Tris, and dialyzed into 10 mM Na phosphate, pH 7.3, 0.15 M NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol.

To prepare Fab, mercuripapain (Sigma Chemical Co., St. Louis, MO) in 70% ethanol was added to the IgG preparation at 20 μl per 10 ml of IgG (10 mg/ml) and digestion was carried out at 37°C for 1 h (19). Papain was inactivated by addition of iodoacetamide to 30 mM for 15 min at 37°C. After dialysis, Fab fragments were separated from Fc fragments by applying the digest to a second column of protein A–agarose. The flow-through fraction, containing Fab, was collected. Purity was checked by gel electrophoresis in SDS.

[125]I-Fab Binding to Intact Platelets

Fab was labeled with ^{125}I, and its binding to platelets was determined at a series of concentrations essentially as described by Pidard et al. (20) except platelets were prepared by gel filtration as in the adhesion assay.

Antisera against Fibronectin, Fibrinogen, and Factor VIII/von Willebrand Factor

High-titer rabbit antisera against human fibronectin and human fibrinogen were obtained from Calbiochem-Behring Corp. (La Jolla, CA). The antisera gave strong reactions on immunodiffusion with purified antigens. Rabbit antiserum against Factor VIII/von Willebrand factor was the gift of Dr. C. Hougie, University of California, San Diego. Fab fragments of IgG from these antisera were prepared as described above.

Preparation of Outdated Platelet Extracts

Outdated human platelet packs were obtained from the Veterans Administration Hospital Blood Bank, La Jolla, and the San Diego Blood Bank. Red blood cell contamination was reduced by repeatedly centrifuging at 500 g for 2 min in a Sorvall GS-4 rotor (DuPont Instruments, Sorvall Biomedical Div., Newtown, CT) equilibrated at 10°C, and discarding the pellet. Platelets were pelleted by centrifugation at 3,000 g for 15 min, resuspended in Tris saline (15 mM Tris-HCl, pH 7.4, 126 mM NaCl, 5.4 mM KCl, 5×10^{-5} M $CaCl_2$, 1 mM $MgCl_2$, 0.1% glucose) and washed three to four times, until the supernatant did not contain appreciable protein, as measured colorimetrically (21). Platelets were then extracted by one of the following methods:

FREEZE-THAW EXTRACTION OF INTACT PLATELETS: Platelets were resuspended in Tris saline without EDTA, and the platelet suspension was frozen and thawed three times (22). Solubilized material was collected by centrifugation at 100,000 g for 1 h at 4°C. The supernatant was collected and used in subsequent fractionations.

OCTYLGLUCOSIDE EXTRACTION OF INTACT PLATELETS: Platelets were resuspended in Tris saline containing 1% octylglucoside and 2 μg/ml phenylmethylsulfonyl fluoride, and were incubated for 1 h at room temperature on a shaker. Centrifugation was performed as above. To test for neutralizing activity of the supernatant in the adhesion assay, octylglucoside was removed by exhaustive dialysis against Tris saline, pH 7.4. The dialyzate was then clarified by centrifugation at 10,000 g in a Sorvall centrifuge.

CHYMOTRYPSIN TREATMENT OF INTACT PLATELETS: The platelet suspension was incubated with 0.4 U/ml insolubilized α-chymotrypsin (Sigma Chemical Co.) for 2 h at room temperature on a shaker. Platelets and enzyme-derivatized beads were removed by centrifugation at 750 g. Phenylmethylsulfonyl fluoride was added to neutralize any residual protease activity, and the extract was clarified at 100,000 g as described above.

Assay of Antibody Effects on Adhesion and Their Neutralization

Antibody effects on platelet–collagen adhesion were determined by preincubating the platelets in assay medium containing a series of concentrations of immune Fab for 10 min at 37°C and then performing the adhesion assay over a 10-min period. To test platelet extracts for neutralization of the effects of immune Fab on platelet–collagen adhesion, a series of concentrations were reacted with antibody that was at a concentration that inhibited ~50% of platelet–collagen adhesion. In general, antiplatelet Fab, at 15 μg/ml, was preincubated with serial dilutions of platelet extract for 30 min at 37°C. ^{51}Cr-labeled human platelets were then added and the tubes incubated for 10 min in a 37°C water bath. Aliquots of 0.3 ml were added to triplicate Linbro wells containing collagen-derivatized coverslips. Platelet adhesion was assayed over a 10-min period as described and quantified by measuring ^{51}Cr bound to the coverslip. Neutralization of adhesion-blocking antibodies was calculated as described by Brackenbury et al. (23) for each dilution of an extract: % neutralization = % adhesion (Fab + extract) − % adhesion (Fab alone)/% adhesion (extract alone) − % adhesion (Fab alone) × 100. One unit of neutralizing activity was defined as that amount that gave 50% neutralization of the adhesion-blocking antibody under defined conditions. It was estimated by plotting the log of extract protein concentration against the percent neutralization for serial assay points and fitting a line by the least-squares method (24). Specific activity was obtained by dividing activity, in units per milliliter, by the extract protein concentration (21), in milligrams per milliliter.

Estimation of Platelet Lysis

Because apparent inhibition of adhesion could result from platelet lysis during the assay, with release of ^{51}Cr, we evaluated lysis at the end of the assay incubation. The supernatant containing unbound platelets and any free ^{51}Cr was collected and lightly fixed with 0.5% paraformaldehyde in 0.03 M Na phosphate buffer, pH 7.4 (17) for 10 min. An aliquot was then filtered over a 0.2-μm filter (Millipore Corp., Medford, MA), and free ^{51}Cr in the filtrate was determined. This was compared to total ^{51}Cr in an unfiltered aliquot. Under standard assay conditions, lysis estimated by this method was 0.3% or less, and was not affected by addition of immune Fab.

Fractionation of Platelet Extracts on Lectin Columns

The platelet extract was applied to a 3-ml column of wheat germ agglutinin–agarose (Vector Labs, Burlingame, CA), equilibrated in 0.05 M Tris-HCl, pH 7.4, containing 0.15 M NaCl. After washing in this buffer, specific elution was carried out with 0.1 M N-acetyl-D-glucosamine in this buffer. The fraction eluted from the wheat germ column W_{bd}, was dialyzed into 0.05 M Tris-HCl, pH 8, containing 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and was chromatographed on a 2-ml column of concanavalin A–agarose (E-Y Laboratories, San Mateo, CA). The flow-through ($W_{bd}C_{ft}$) was saved, and this column was eluted with 0.1 M α-methyl mannoside in the same buffer to give rise to the fraction $W_{bd}C_{bd}$. All fractions were dialyzed, lyophilized, and resuspended in Tris saline to assay neutralizing activity.

Preparation of Columns of Partially Purified Platelet Extract, and Partially Purified Antiplatelet IgG

To partially purify the components of antiplatelet IgG that bound to entities that influenced platelet–collagen adhesion, we prepared a fraction enriched in platelet proteins that did not neutralize the adhesion-blocking activity of the antiserum and used this to adsorb irrelevant antiplatelet antibodies. To this end, an aliquot of an octylglucoside extract of whole platelets was repeatedly passed through columns of wheat germ agglutinin–agarose, and the final flow-through fraction, W_{ft}, was collected. An aliquot of W_{ft} containing ~20 mg of protein was coupled to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals) at 4°C (25). The gel was postincubated with 1 M ethanolamine, pH 9, to block unreacted residues. Antiplatelet IgG was then reacted with this column by circulating 10 mg IgG in phosphate-buffered saline, pH 7.2, over W_{ft} agarose for 24 h at 4°C (26). The flow-through fraction, enriched in antibodies that blocked platelet–collagen adhesion, was collected, coupled to cyanogen bromide-activated Sepharose, and used to purify the platelet antigens with which it reacted. To recover the antibodies that bound to the W_{ft} agarose column, it was washed (26) and specifically eluted with 0.2 M diethylamine, pH 11.5. The IgG fractions that flowed through and bound to the W_{ft} agarose were digested to Fab fragments, as above, to assess their binding to platelets and their effect on platelet–collagen adhesion.

PAGE

Samples were solubilized by boiling for 10 min in 0.2 M Tris-HCl, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol, and 10% glycerol and electrophoresed in 7.5% polyacrylamide slab gels in 0.1% SDS in 0.1 M Tris–glycine buffer, pH 8.2.

Prestained standards of M_r 200,000, 92,000, 66,000, 43,000, and 26,000 (Bethesda Research Laboratories, Gaithersburg, MD) were run in parallel lanes. Proteins were localized by silver staining (27). In some experiments in which neutralizing activity was studied, unfixed gels were sliced into appropriate zones and protein eluted by overnight incubation in SDS-containing buffer, and freed of SDS by addition of acetone as described by Hager and Burgess (28). Bovine serum albumin (radioimmunoassay grade, Sigma Chemical Co.), 0.1 mg/ml, was used as a carrier protein. The eluted protein in each fraction was resolubilized in 40 μl of 6 M guanidine hydrochloride, diluted 1:50 with Tris saline, and dialyzed extensively against Tris saline. Serial dilutions were tested in the platelet–collagen adhesion assay for antibody neutralizing activity.

RESULTS

Fab prepared from antiplatelet antiserum completely blocked platelet–collagen adhesion in a dose-dependent manner (Fig. 1). Preimmune Fab had no effect (Fig. 1). Fab directed against fibronectin, fibrinogen, or Factor VIII/von Willebrand's factor, implicated in other platelet functions also had no detectable effect on our assay, even at 10- to 20-fold higher concentrations.

To determine the nature of the materials with which the antibody reacted, we extracted the platelet constituents in three ways—by freezing and thawing, by treatment with the nonionic detergent octylglucoside, or by treating the surface of the platelets with a protease, α-chymotrypsin. Each of these extracts could completely neutralize the adhesion-blocking Fab, at the appropriate concentration which, with the octylglucoside extract, was ~350 μg/ml (Fig. 2). The octylglucoside extract seemed preferable to the others as the starting material for further purification inasmuch as the freeze-thaw extract had a lower specific activity, and the α-chymotrypsin extract had a lower total yield (Table I). The latter also seemed undesirable because the antigen it contained might be a proteolytic fragment rather than an intact molecule. The freeze-thaw procedure may also activate proteases and give rise to cleaved platelet glycoproteins (29), notably the membrane glycoprotein, glycoprotein Ib.

Fractionation was then attempted by affinity chromatography on wheat germ lectin–agarose. The neutralizing activity
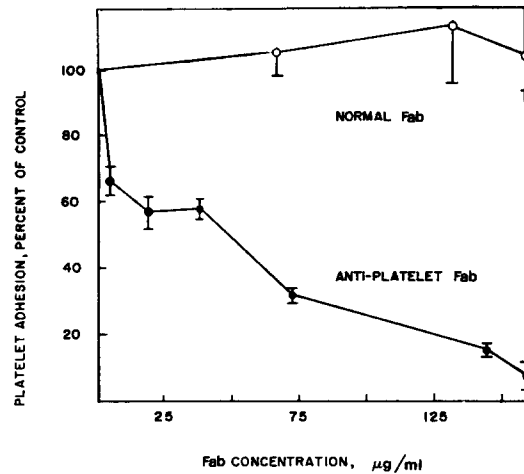

FIGURE 1 Effect of normal (O; preimmune) and antiplatelet rabbit Fab (●) on platelet–collagen adhesion. Fab was preincubated with ^{51}Cr-labeled human platelets for 10 min at 37°C and the adhesion of the platelets to immobilized trimeric collagen was determined as described in Materials and Methods. Each point represents the mean ± SE of triplicate determinations.
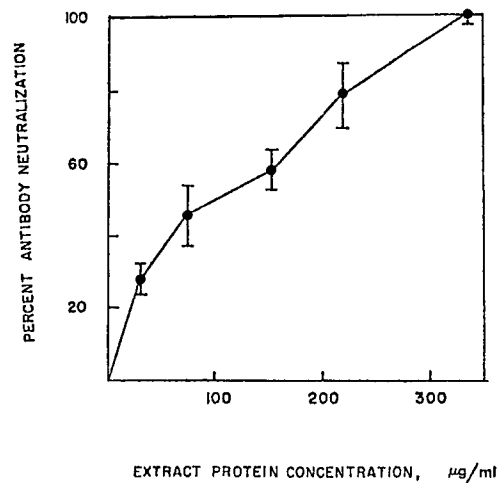

FIGURE 2 Neutralization of adhesion-blocking Fab by platelet extract. Dilutions of a dialyzed octylglucoside extract of human platelets were preincubated with antiplatelet Fab as described in Materials and Methods, and the effects of the mixture on platelet–collagen adhesion was determined. Results are mean ± SE of triplicate determinations. Parallel samples were incubated with preimmune Fab and octylglucoside extract, and showed that adhesion was not directly affected by the extract.

in both the octylglucoside extract and the freeze-thaw extract bound virtually completely to this column and could be eluted with N-acetyl-D-glucosamine (Table II). With both extracts, the small amount of neutralizing activity that flowed through the column was completely bound when once more applied to the column. The specific activity of the material purified from the octylglucoside extract was ~30-fold greater than that of the initial extract and that from the freeze-thaw extract was ~20-fold greater.

We then sought to subfractionate the wheat germ bound neutralizing material (designated W_{bd}) derived from the octylglucoside extract, by affinity chromatography on concanavalin A–agarose. Some of the neutralizing activity flowed through the concanavalin A column (designated $W_{bd}C_{ft}$) and could not bind even with repeated passage. Another fraction

TABLE I

Extraction of Antibody-neutralizing Activity from Whole Platelets

Extraction method	Protein extracted*	Specific activity
	mg	U/mg protein
Freeze-thaw	1.12	5.2
Octylglucoside	0.49	9.6
α-Chymotrypsin	0.11	28.8

Outdated human platelets were extracted and neutralizing activity was calculated, as described in Materials and Methods.
* Yield per 100 ml of blood. Starting material consisted of outdated human platelet packs.

TABLE II

Fractionation of Neutralizing Activity on Wheat Germ Lectin-Agarose

Fraction	Protein re-covered	Neutral-izing activity	Specific activity
	% of applied	% of applied	U/mg protein
1% Octylglucoside extract			
Flow-through fraction	70	3	0.4
Bound and eluted fraction	5	89	291
Freeze-thaw extract			
Flow-through fraction	72	10	0.7
Bound and eluted fraction	4.3	82	99.2

Outdated human platelets were extracted as described, and fractionated on wheat germ lectin–agarose. Neutralizing activity was calculated as described in Materials and Methods.

bound to concanavalin A (designated $W_{bd}C_{bd}$) and was eluted with α-methylmannoside. To our surprise, neither fraction was capable of completely neutralizing the Fab, even at high concentrations (Fig. 3), although the starting material that had been eluted from the wheat germ lectin column neutralized completely. This was not due to protein losses on the column, because subsequent elution at low pH or with SDS did not release neutralizing activity. We consistently found that $W_{bd}C_{ft}$ was more potent than $W_{bd}C_{bd}$. Under standard conditions, in which neutralization was assayed at a Fab concentration that blocked 50% of platelet–collagen adhesion, the former fraction neutralized up to ~58% of the Fab and the latter up to ~25% (Fig. 3). This was the maximum percent neutralization achieved with each fraction, and the addition of larger amounts of the fraction produced no further neutralization (Fig. 3).

Because each of these fractions only neutralized partially under standard conditions (Fab at a concentration that inhibits 50% of the platelet–collagen adhesion), we analyzed the relative potency of the two subfractions and that of W_{bd} over a wide range of Fab concentration. In each experiment, a Fab concentration was chosen that inhibited platelet–collagen adhesion between 40–100% based on dose-response curves prepared previously, and a series of concentrations of the fraction of interest were tested for neutralizing activity. Maximal neutralization was determined as in Fig. 3. We found that W_{bd} could, at an appropriate concentration, completely neutralize the Fab, irrespective of the degree of inhibition of adhesion by Fab (Fig. 4). In contrast, the two subfractions, each of which partially neutralized at Fab concentrations that inhibited platelet–collagen adhesion in the 50% range, had
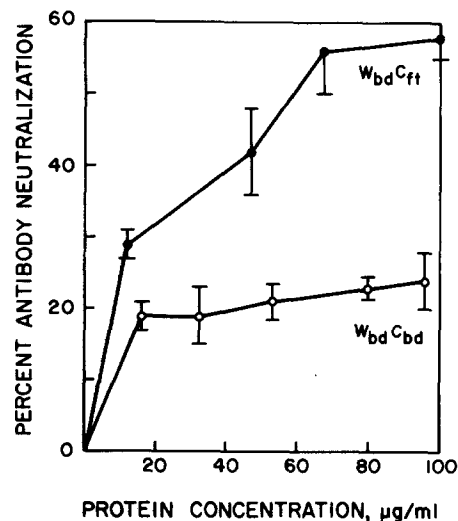

FIGURE 3 Asymptotic neutralization of adhesion-blocking Fab by subfractions of a platelet extract. The W_{bd} fraction of the platelet octylglucoside extract was subfractionated on a concanavalin A–agarose column and the neutralizing effects of a series of concentrations of the two subfractions, $W_{bd}C_{ft}$ (●) and $W_{bd}C_{bd}$ (○), were determined. Results are the mean ± SE of these determinations.
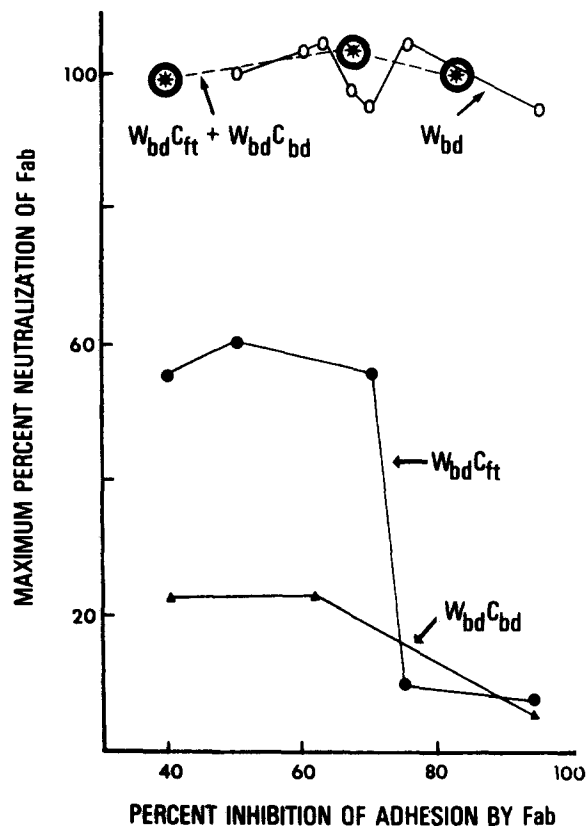

FIGURE 4 Maximum neutralizing activity of fractions of a platelet extract as a function of inhibition of platelet–collagen adhesion by a series of dilutions of antiplatelet Fab. The fractions W_{bd}, $W_{bd}C_{bd}$, and $W_{bd}C_{ft}$ were prepared from an octylglucoside extract of human platelets by lectin-affinity chromatography. A recombined fraction containing equal amounts of $W_{bd}C_{bd}$ and $W_{bd}C_{ft}$ was also tested. Maximum neutralization of Fab at each dose of Fab was determined as in Fig. 3.

no significant neutralizing activity when tested at Fab concentrations that inhibited close to 100% (Fig. 4). However, a mixture of the two fractions could, like the parent fraction, neutralize totally, even at Fab concentrations that inhibited platelet–collagen adhesion very extensively (Fig. 4). The implications of these findings will be considered later.

Because $W_{bd}C_{ft}$ was the more potent subfraction, we further fractionated it by PAGE in SDS. The fractions were eluted from the gel and their neutralizing activity was determined under standard conditions in which Fab concentrations that inhibited 50% of platelet–collagen adhesion were employed. Two active fractions were resolved by this procedure. One with $M_r \sim 90,000$–$100,000$ produced maximum neutralization of $\sim40\%$ (Table III). Another with $M_r \sim 110,000$–$130,000$ produced maximal neutralization in the range of 20%. This latter fraction was initially isolated from two slices of the gel (Table III), but probably represents a single material that runs in both fractions, inasmuch as combining the contents of the two fractions gave no greater maximum neutralization than was observed with each alone. This electrophoretic procedure apparently resolves two antigenically distinct entities in the $W_{bd}C_{ft}$ fraction, so that together with $W_{bd}C_{bd}$, three distinct neutralizing fractions apparently exist. Their relationship will be considered later.

Electrophoretic analysis of W_{bd}, $W_{bd}C_{ft}$, and $W_{bd}C_{bd}$ by PAGE in SDS under reducing conditions revealed many protein bands that reacted with a silver stain (not shown). Because all these fractions were so impure, we turned to an alternative purification approach, by taking advantage of the fact that the portion of the octylglucoside extract that failed to bind to a wheat germ lectin column (W_{ft}) had no detectable neutralizing activity. We, therefore, coupled W_{ft} to agarose and exhaustively adsorbed an IgG fraction prepared from our crude antiserum on this column. Unbound IgG retained adhesion-blocking activity, but many other antiplatelet antibodies were removed by the column. We then used the IgG preparation enriched in neutralizing activity as an affinity

adsorbant by covalently linking it to agarose. Extract constituents that had neutralizing activity should bind to this column, although many other platelet constituents might well also bind.

The fraction of W_{bd} that bound to this column and was specifically eluted at pH 11.5 contained only three major protein bands upon PAGE (Fig. 5). It will be referred to as "enriched neutralizing antigen." The very prominent band with $M_r \sim 125,000$ (Fig. 5, arrow) migrated exactly with a purified preparation of platelet glycoprotein IIb and the prominent band below it with $M_r \sim 100,000$ co-migrated with platelet glycoprotein IIIa. The enriched neutralizing antigens neutralized antiplatelet Fab effects on platelet–collagen adhesion in a dose-dependent manner up to a maximum of $\sim50\%$ inhibition (Fig. 6).

Given the fact that none of the subfractions prepared from W_{bd} neutralize Fab completely, it is difficult to compare formally their relative potencies and specific activities with each other or with the starting fraction, W_{bd}. The data are summarized in Table IV. Here we show maximum neutralization achieved with each subfraction under standard assay conditions (Fab concentration that inhibits 50% of platelet–collagen adhesion), and the protein concentration of each subfraction required to produce half the maximum neutralization achievable with that fraction. Based on these comparisons, it appears that the enriched neutralizing fraction is relatively potent, although it is clearly not capable of neutralizing antibody completely. It is likely that it is enriched in at least one relevant molecule and, based on work with monoclonal antibodies reported in the subsequent article (16), the

TABLE III

Fractionation of Antibody-neutralizing Activity by PAGE in SDS

Eluted zone	Maximum neutralization observed Mean ± SE
Dye front–90	1 ± 5
90–100	40 ± 17
100–110	6 ± 10
110–120	19 ± 3
120–130	23 ± 9
130–140	0 ± 5
140–150	−10 ± 14
150–160	−15 ± 8
160–170	−15 ± 16
170–top of gel	−1 ± 10
Starting material:	
$W_{bd}C_{ft}$	58 ± 8

An octylglucoside extract of whole platelets was reacted with a wheat germ lectin–agarose column and the bound material that had been eluted with N-acetyl-glucosamine was passed through a concanavalin A–agarose column. This material ($W_{bd}C_{ft}$) was subjected to slab gel electrophoresis and the gel was cut into zones and eluted, all as described in Materials and Methods. The zone dye front–90 includes all material between the dye front and apparent $M_r \sim90,000$; the zone 90-100 includes all material with apparent $M_r \sim90,000$–$100,000$, etc. The apparent M_rs are all based on migration of prestained standards run in parallel lanes of the slab gel. Maximum neutralization was measured as in Fig. 3.
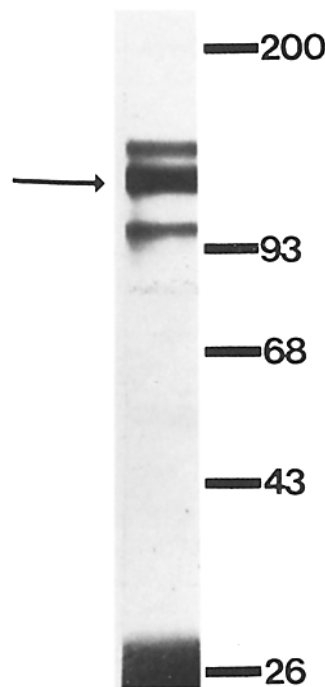

FIGURE 5   PAGE of enriched neutralizing antigens. Electrophoresis was performed as described in Materials and Methods. The gel was stained with silver nitrate (27). Migration of molecular weight standards with $M_r \sim 200,000$, $93,000$, $68,000$, $43,000$, and $26,000$ is indicated. The dye front was slightly below the standard with $M_r \sim26,000$. The arrow indicates the migration of platelet glycoprotein IIb, determined by electrophoresis of a preparation (the kind gift of Dr. E. Plow, Scripps Clinic and Research Foundation, La Jolla, CA) enriched in this protein on a parallel lane of the slab gel.
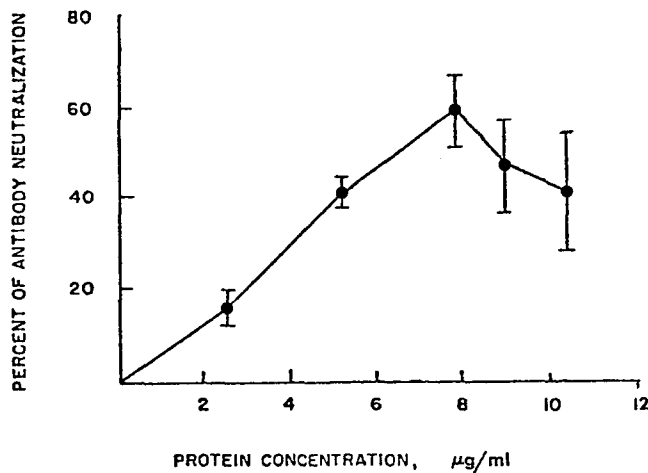
FIGURE 6 Neutralization of antiplatelet Fab by enriched neutralizing antigens. Enriched neutralizing antigens were prepared as described in the text, dialyzed into modified Tyrode's buffer, pH 7.4, and tested in the adhesion assay against antiplatelet Fab, as described in Materials and Methods. Each data point was assayed in triplicate and is expressed as mean ± SE.

molecule with $M_r \sim$ 125,000, which appears to be glycoprotein IIb (30), probably contributes to the neutralizing activity of this fraction.

We also used the antibody that bound to the W_{ft} fraction in another way. Because it had no effect on platelet–collagen adhesion but bound many platelet antigens, we could use it to assess the amount of "nonspecific" Fab that can bind to a platelet without affecting its adhesion to collagen. ^{125}I-labeled Fab prepared from this fraction bound extensively to platelets, saturating at $\sim 2 \times 10^6$ Fab molecules/platelet (data not shown). Under these conditions, platelet–collagen adhesion was normal. In contrast, binding a comparable number of Fab molecules of an active preparation inhibited adhesion by at least 50%.

DISCUSSION

Univalent antibody fragments prepared from a rabbit antiserum raised against whole human platelets completely blocked platelet–collagen adhesion in a quantitative assay. In contrast, univalent antibody fragments directed against fibronectin, fibrinogen, and Factor VIII/von Willebrand's factor had no effect upon platelet–collagen adhesion in our assay, even at concentrations 50–100 times greater than the effective dose for antiplatelet antibody. Thus, although these glycoproteins play important roles in platelet aggregation (12, 14, 31, 32) and antifibronectin (15) or anti-Factor VIII/von Willebrand's factor (8, 9, 32) have been reported to influence platelet behavior in other assays, they do not appear to be required in our assay in which platelet aggregation is minimized.

As in other cell adhesion systems, a crude extract of platelets completely neutralized the adhesion-blocking activity of our crude antiserum. In other adhesion systems (1, 2, 33–36), a single antigenic substance was identified as a CAM, and could completely neutralize the antiserum. What is remarkable about the present experiments is that subfractions of the crude extract contained activity that only partially neutralized the adhesion-blocking antibodies, even at saturating conditions.

The partial neutralizing effects of these subfractions were

highly dependent on the concentration of Fab used in the adhesion assay. At relatively low Fab concentrations that inhibited ~50% of platelet–collagen adhesion, subfraction $W_{bd}C_{ft}$ neutralized up to 60% of the adhesion-blocking activity of the Fab, and subfraction $W_{bd}C_{bd}$ neutralized up to ~25%. These levels of neutralization were maximal, in that adding increasing amounts of either neutralizing fraction to a constant amount of Fab did not produce further neutralization. At high Fab concentrations that inhibited 80–90% of platelet–collagen adhesion, neither subfraction had a statistically significant effect on platelet–collagen adhesion, even if assayed at high concentrations. In striking contrast, a mixture of these two subfractions or the parent fraction, W_{bd}, could totally neutralize Fab at this concentration.

These findings indicate that the $W_{bd}C_{ft}$ and $W_{bd}C_{bd}$ contain immunologically distinct entities, each capable of neutralizing a different subpopulation of adhesion-blocking Fab. Each subpopulation of Fab is apparently capable of completely inhibiting platelet–collagen adhesion inasmuch as complete neutralization of one subpopulation at high Fab concentration has no effect on the ability of the other, unneutralized Fab subpopulation to inhibit all platelet–collagen adhesion.

The dependence of the neutralizing effect of the different subfractions on the Fab concentration suggests that all of the active molecules detected here function in a single, dependent pathway leading to platelet–collagen adhesion, rather than mediating parallel, independent types of adhesion. If the two fractions did represent entities that mediated independent types of adhesion, each should neutralize the complex Fab mixture up to the same saturation level irrespective of the Fab concentration used, provided enough is added to bind all the Fab that reacts with it. Yet, the data indicate that even high concentrations of either $W_{bd}C_{ft}$ or $W_{bd}C_{bd}$ are completely unable to neutralize high concentrations of the complete Fab. This is consistent with the idea that molecules contained in $W_{bd}C_{ft}$ and $W_{bd}C_{bd}$ participate in a dependent adhesion pathway rather than in independent ones. If enough Fab is added to fully block either the relevant components of $W_{bd}C_{ft}$ or of $W_{bd}C_{bd}$ on the platelet, complete inhibition of adhesion results. Neutralizing the Fab directed against one component does not restore adhesion as long as the other component remains blocked.

TABLE IV

Comparisons of the Activity of Various Neutralizing Fractions

Fraction	Maximum neutralization	Protein required for half-maximum neutralization	Protein required for maximum neutralization
	%	μg/ml	
Octylglucoside extract	100	104	300
W_{bd}	100	10	36
$W_{bd}C_{ft}$	58	13	65
$W_{bd}C_{bd}$	24	10	48
Enriched neutralizing antigen	50	4	7

Summary of measures of the relative neutralizing activity of a platelet extract and fractions derived from it. Maximum neutralization for each fraction (i.e., the maximal percent neutralization that saturating concentrations of this fraction can produce, when assayed against Fab concentrations that inhibit 50% of adhesion) was determined as in Fig. 3. This became the basis for further comparisons. The concentration of protein required to produce half maximum neutralization and the minimal concentration that produced maximum neutralization is tabulated.

This interpretation is completely consistent with results obtained with a monoclonal antibody in the subsequent article (16). That antibody, although apparently directed against a single platelet glycoprotein, can totally inhibit platelet-collagen adhesion. Yet the antigen to which it is directed only partially neutralizes the polyclonal Fab used here, to a maximal level of ~25% (16). This again suggests that several platelet entities participate in platelet-collagen adhesion, and that they are not independent.

It remains possible that the multiple adhesion determinants discovered in this assay might represent protease fragments of a single adhesion molecule, each containing unique antigenic sites. Octylglucoside extraction of platelets may result in the release of proteases which could, despite phenylmethylsulfonyl fluoride treatment, degrade adhesion molecules into smaller fragments. However, it would be necessary for the degradation to be complete, with no survival of the parent molecule, to lead to the results we found, which seems very unlikely.

The subfraction $W_{bd}C_{ft}$ can be further resolved into two subfractions by PAGE in SDS. Again, the two subfractions resolved by this procedure appear to be antigenically distinct because each is capable of neutralizing antibody only up to a maximum level that is considerably lower than that of the parent fraction. The smaller of the two active subfractions does not appear to be a degradation product of the larger active subfraction because, were this the case, the larger would be expected to be as effective a neutralizer as the starting material, $W_{bd}C_{ft}$. It is possible that both fractions are fragments of a single, even larger molecule which contains both groups of antigenic determinants. However, were this the case, one would have to conclude that the parent molecule was either completely degraded into these two fragments during fractionation, or was so large that it did not enter the polyacrylamide gel, because no active neutralizing material was found with M_r greater than ~130,000. It seems more likely that the two subfractions resolved by electrophoresis are distinct antigenic entities and possibly are distinct molecular entities, each playing a different but interdependent role in platelet-collagen adhesion, following reasoning like that detailed above. Whether the antigenically distinct entities represent distinct posttranslational modifications of one molecule or fundamentally different molecules that play different roles in platelet-collagen adhesion cannot be determined from this analysis. The fact that N-CAM exists in different forms, based on posttranslational modifications (1) provides a precedent for this possibility.

Given the apparent complexity of the system, we sought a way to separate the relevant components by first selectively purifying the crude antibody preparation and then using it to further purify the subfractions that had been resolved by lectin-affinity chromatography. This procedure gave rise to an active enriched neutralizing fraction that contained a relatively small number of protein bands, including a prominent protein with $M_r \sim 125,000$. This fraction could form the basis for further rounds of immunization and purification, following the "iterative approach" (1). An alternative was to turn to monoclonal antibodies to help resolve this complex situation. In the following article (16), we describe the utility of monoclonal antibodies in identifying one component of this adhesion mechanism.

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