Platelet Aggregation by Fibrinogen Polymers Crosslinked Across the E Domain

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There is evidence that platelet interactions with artificial surfaces are mediated by plasma proteins, especially fibrinogen, adsorbed on the surfaces. Multiple site interactions between fibrinogen molecules adsorbed in high concentration and receptors in the unactivated platelet may be sufficient for platelet adhesion and subsequent activation. To examine this hypothesis, we prepared soluble polymers of fibrinogen. Polymers produced by interaction of fibrinogen with Fab', fragments of antibodies against fibrinogen’s E (central) domain (Fg-Fab', E) induced, in gel-filtered platelets, aggregation and serotonin release, which were blocked by monoclonal antibodies against the GPIIb/IIIa complex, by Fab fragments against the D domain, and by metabolic inhibitors; aggregation was attenuated but not abolished by enzymatic removal of ADP (with CP/CPK) or by blockage of ADP binding sites (with FSBA), and when secretion was inhibited by aspirin. Fg-Fab', E also induced a dose-dependent elevation in cytoplasmic Ca²⁺ (measured by Aequorin luminescence) which was attenuated by CP/CPK and by FSBA, and was eliminated by metabolic inhibitors and by anti-IIb/IIIa antibody. Fibrinogen complexes crosslinked with dimethylsuberimidate or Factor XIII neither aggregated gel-filtered platelets nor inhibited platelet aggregation by ADP and fibrinogen, probably because of inaccessibility of lysine residues in the D (terminal) domain of fibrinogen, which are thought to be required for platelet binding. Thus, soluble complexes of fibrinogen having multiple available platelet receptor recognition sites activate gel-filtered platelets and may provide a useful model for platelet-surface interactions mediated by adsorbed fibrinogen.

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When an artificial surface is exposed to blood, it rapidly acquires an adsorbed film of plasma proteins, which precedes the interaction of the surface with blood cells, including platelets.1-4 Considerable evidence supports the hypothesis that adsorption of a specific protein, fibrinogen (Fg), by artificial surfaces is a prerequisite for subsequent platelet adhesion.5-10 Brash et al11-12 observed that fibrinogen is often preferentially adsorbed from mixtures with albumin or gammaglobulins and that the fibrinogen-to-albumin ratio in the adsorbed layer correlates with platelet reactivity induced by many surfaces. Collier13 reported that latex particles coated with fibrinogen agglutinate gel-filtered platelets (GFP) without the addition of other platelet agonists. In our laboratory in vitro studies of platelet retention on bead columns have also indicated that although albumin precoating of the beads reduces platelet reactivity (retention), fibrinogen precoating markedly augments it.14

On the other hand, platelets are normally bathed in a fibrinogen solution in the blood but do not bind appreciable quantities of fibrinogen in blood in vitro, and presumably not in the circulation in vivo, unless they are stimulated by agonists such as adenosine diphosphate (ADP), thrombin, or epinephrine.15-19 The receptor for fibrinogen on stimulated platelets has been identified as the glycoprotein IIb/IIIa complex.20-24 Hawiger et al25-28 and Plow and associates29,30 have suggested that a locus on the carboxy-terminal region of the fibrinogen gamma chain is the primary binding site for this receptor complex. The possibility that fibrinogen may interact with two classes of platelet receptors, those with high or low affinity, has been suggested.19,31,32

Why platelets require activation before reacting with fibrinogen in fluid blood but apparently not with fibrinogen adsorbed on a surface has been puzzling. A popular explanation is that platelet interactions with surface-adsorbed fibrinogen result from an alteration in the conformation of the fibrinogen molecule as a result of its adsorption on the surface. Conformational changes in fibrinogen adsorbed on various surfaces have in fact been described.33-36 However, Lindon et al37 have shown that platelet interaction with fibrinogen-coated polyalkyl methacrylate surfaces correlated with the concentration of antibody-detectable or “native” fibrinogen on the surface rather than with the total amount of fibrinogen bound, suggesting that preservation of recognizable platelet-biding loci on adsorbed fibrinogen molecules may be important in platelet adhesion. Similarly, Chuang et al38 have suggested that variable retention of the conformational integrity of adsorbed fibrinogen may explain differences in platelet adhesion to surfaces of dialysis devices.

It is accepted that fibrinogen acts as a cofactor in platelet aggregate formation after the activation of the platelets by ADP or other agonists has resulted in exposure of the glycoprotein IIb/IIIa receptors. However, it is also possible that fibrinogen molecules arrayed on a surface may themselves activate platelets and facilitate platelet adhesion. When adsorbed on a surface, proteins may be present in greatly increased local concentration: e.g., a surface that has adsorbed 1 µg Fg/cm² has a surface fibrinogen concentration equivalent to that of a solution containing 220 mg/ml.39 Furthermore, closely arrayed molecules adsorbed on a surface may simultaneously present a multitude of specific binding sites for cell membrane receptors. Although individual sites may interact only weakly with “low affinity” receptors, this “multivalency” of available sites may result in binding which in the aggregate is quite strong.39,40 Finally,

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adsorption on a surface may induce conformational changes in a protein that may either enhance or reduce the accessibility or recognition of a binding site.\textsuperscript{13,36}

As a way of testing the possible effects that high local concentrations of fibrinogen with multiple platelet-binding loci may have upon platelets, we sought to prepare soluble polymers of fibrinogen that had these characteristics. These polymers, not to be confused with fibrin, might be considered as models of fibrinogen adsorbed on an artificial surface. Although these soluble polymers might not be identical in conformation to fibrinogen that has become surface-bound, the multivalent presentation of platelet binding loci may mimic events that take place when circulating platelets interact with protein-coated surfaces.

**MATERIALS AND METHODS**

**Materials.** Human fibrinogen and plasmin were obtained from Kabi, Helena Laboratories (Beaumont, Tex). Human fibrinogen was also obtained from US Biochemicals (Cleveland, Ohio) as were goat Fab\textsubscript{g} fragments specific for rabbit IgG heavy and light chains. Sepharose 2B and 4B, Sephadex G-25 and G-75, and Sephacryl S-200 and S-500 were purchased from Pharmacia (Piscataway, NJ). From Pierce Chemical (Rockville, Ill), dimethylsuberimidate and lodogen were obtained. Parke-Davis (Morris Plains, NJ) was the supplier for human thrombin. Worthington (Freehold, NJ) provided papain and pepsin. The molecular weight standardization kits for SDS-PAGE were obtained from Biorad (Rockville Center, NY) and Sea-gamma-alumiga gel, L-phenylalanyl-L-prolyl-L-arginyl-chloromethylketone (PPACK), and Freund's adjuvant were purchased from Calbiochem (La Jolla, Calif). Epsilon amino caproic acid, benzamidine hydrochloride, triethanolamine, HEPES, bovine serum albumin, aprotinin, apyrase, phosphocreatine, creatine phosphokinase, 5'-fluorosulfonylbenzoyladenosine (FSBA), fetuin-agarose, lysine-agarose, Protein A-Sepharose, and 2-deoxyglucose were obtained from Sigma (St. Louis, Mo). DEAE-Sepacel, CM-Sepharose, Sepharose 2B and 4B, Sephadex G25 and G75, and Sephacryl S-200 and S-500 were purchased from Pharmacia (Piscataway, NJ). From Pierce Chemical (Rockville, Ill), dimethylsuberimidate and lodogen were obtained. Parke-Davis (Morrism Plains, NJ) was the supplier for human thrombin. Worthington (Freehold, NJ) provided papain and pepsin. The molecular weight standardization kits for SDS-PAGE were obtained from Biorad (Rockville Center, NY) and Bethesda Research Laboratories (Gaithersburg, Md). Aequorin was obtained from Dr John Blinks, Mayo Clinic, Rochester, Minn. Murine monoclonal antibodies in ascites fluid were generously donated by Dr Barry Coller (Dept of Hematology, State University of New York at Stony Brook, NY). Outdated citrated plasma was a gift of the Beth Israel Hospital blood bank.

**Preparation of human fibrinogen.** Human fibrinogen was prepared from pooled outdated citrated plasma or from partially purified commercial products by a modification of the method reported by Wang.\textsuperscript{41} Citrated human plasma was stirred slowly (4°C) with a slurry of alumina gel sufficient to increase the prothrombin time to greater than 70 sec (~500 mg gel/100 mL plasma). After centrifugation (20,000 g, 30 min, 4°C) the supernatant was decanted. When commercial human fibrinogen was employed, it was initially dissolved in buffer B (55 mmol/L sodium citrate, 100 mmol/L epsilon-amino caproic acid, and 10 mmol/L benzamidine HCl). The subsequent purification scheme was similar for commercial and plasma sources of fibrinogen. Precipitation with lysine (to 0.2 mol/L) and glycine (to 2.0 mol/L) was performed with slow stirring (4°C, 3 hr) after which the precipitate was recovered by centrifugation (20,000 g, 30 min, 4°C). The precipitate was repeated after dissolving the pellet in BEC (8 mg/mL). After recovery the precipitate was dissolved in PCBS (21 mmol/L sodium phosphate, pH 6.5, 72 mmol/L sodium chloride, 0.5 mmol/L KCN), dialyzed (4°C, 24 hr), and centrifuged (25,000 g, 1 hr, 4°C), and the supernatant was passed over lysine-agarose and eluted with PBS (0.1 mol/L sodium phosphate, pH 7.4). The fibrinogen solution was concentrated by lyophilization and redissolved in 0.3 mol/L NaCl (15 to 20 mg/mL). Concentration was measured by spectrophotometric absorbance at 280 nm using an extinction coefficient of 15.3. After exhaustive dialysis against 0.3 mol/L NaCl, the solution was stored in aliquots at ~80°C. Clotting time was >96%. Examination by reduced SDS-PAGE (method of Laemmli\textsuperscript{42}) revealed a doublet A-alpha band of M, 68,000, singlet B-beta band of M, 54,000, and singlet gamma band of M, 47,000. No significant contaminants were seen; ie, <1% Factor VIII:WF, fibronecin, and fibrin.

**Preparation of fibrinogen D and E fragments.** Plasmin digestion of fibrinogen was performed in the presence of calcium chloride (50 mmol/L) and PPACK (1 mmol/L) according to previously described procedures.\textsuperscript{21,24} Purity of fragments was verified by nonreduced SDS-PAGE on linear gradient gels (3% to 15%) which revealed a single band (M, 94,000) for the D fragment and a single band (M, 48,000) for the E fragment.

**Preparation of antihuman fibrinogen antibodies.** Healthy male rabbits were injected intramuscularly with human fibrinogen (500 μg) in Freund's adjuvant (50 μL), followed by a second injection 4 weeks later. Booster immunizations were given intramuscularly at 4-week intervals. Three to seven days following booster administration the rabbits were bled from the central artery of the ear. The serum IgG fraction was removed by ammonium sulfate precipitation (42% saturation) and then purified by affinity chromatography using columns of Fg-Sepharose, fragment-D-Sepharose, and fragment E-Sepharose, prepared according to the procedure of Cuatre-casas.\textsuperscript{49} Antibodies were separated into fractions recognizing fibrinogen and E fragment (but not D), and fibrinogen and D fragment (but not E). Cross-reactivity of antibodies for D fragment and E fragment was assessed by Western gel blotting techniques\textsuperscript{49,50} and was not detectable. The fragment-specific antibodies were dialyzed against distilled water, lyophilized, and stored at ~80°C.

**Preparation of fibrinogen polymers.** Preparation of Fg-DMS polymer by crosslinking with dimethylsuberimidate (DMS). DMS was dissolved (100 mmol/L) in triethanolamine (0.5 mol/L) at pH 8.5. Fibrinogen was diluted to 5 mg/mL in 10 mL of triethanolamine (0.5 mol/L, pH 8.5). At 37°C, DMS was added in 500 μL portions to the fibrinogen solution and rapidly mixed. After five minutes, 4 mL of 500 mmol/L ammonium acetate was added to quench the unreacted DMS, the mixture was centrifuged at 20,000 g for 15 minutes, and the supernatant was rapidly exchanged into 0.3 mol/L NaCl by passage through a column of G25 Sephadex. The products of reaction were electrophoresed under nonreducing conditions with sodium dodecyl sulfate (SDS) on 3% polyacrylamide slab gels according to the method of Laemmli\textsuperscript{42} and stained with Coomassie blue. Estimation of relative molecular weights based upon relative band mobilities indicated that a distribution of covalently associated fibrinogen oligomers had been formed, with the largest distinguishable species being pentameric. Dimeric and trimeric oligomers predominated. Clottability of the unfractionated population of Fg-DMS oligomers was 60%, a marked decrease from that of the fibrinogen monomer precursor.

**Preparation of fibrinogen cross-linked by activated factor XIII (Fg-F13).** Factor XIII was purified from human plasma according to the procedure of Kazama et al\textsuperscript{20} and employed to crosslink fibrinogen monomers by the method of Kanaide et al.\textsuperscript{48} Calcium chloride (3.2 mmol/L) and human thrombin (10 U/mL) were added to Factor XIII (0.8 mg/mL) in 0.1 mol/L sodium phosphate, pH 7.4 (PBS), and incubated (37°C, 20 min). PPACK (10 μmol/L) was then added, vigorously mixed, and incubated (37°C, 10 min), after which the mixture was centrifuged (10,000 g, 15 min, 20°C). To the supernatant, calcium chloride (15 μmol/L) and purified fibrinogen (6 mg/mL) were added; the mixture was incubated (37°C, 60 min).
and EDTA added (8 mmol/L). After rapid exchange into PBS using G-25 Sephadex, the unfractonated mixture was stored at -80 °C. Clottability of the unfractonated mixture was approximately 85%. Analysis on nonreduced 3% SDS-PAGE revealed a distribution of fibrinogen polymeric species with predominantly dimers and trimers and detectable amounts of tetramers and pentamers. Clottability of this population of Fg-F13 oligomers was 85%.

Preparation of Fg-Fab(E) (two components) and Fg-Fab(E)-Fab’ (three components) fibrinogen polymers by crosslinking with anti-fibrinogen antibody fragments. Preparation of fibrinogen polymers with E-domain-specific antibodies was undertaken out of concern that alteration of native fibrinogen loci, necessary for binding to platelet receptors for fibrinogen, might occur during nonspecific chemical or enzymatic crosslinking. Monovalent (Fab) and divalent (Fab2) antibody fragments were prepared using procedures of Porter and Nisonoff as previously described. Fab-E polymer was prepared using E-fragment-specific Fab and fibrinogen in a 2:1 molar ratio, which resulted in less than 2% precipitation at 22 °C. Fg-Fab(E)-Fab’ polymer was prepared by mixing antifibrinogen Fab (produced in rabbit), goat Fab (against rabbit Fab), and fibrinogen in a 2:3:1 molar ratio. Less than 10% of the Fg-Fab(E)-Fab’ polymer precipitated at 22 °C.

Passage of the Fg-Fab(E) polymer over a Sepharose 4B size exclusion chromatography column produced a single peak at the void volume, indicating that the apparent molecular size of the complex was equal to or greater than that of a globular protein with M, 20 × 10³ or a polysaccharide chain of M, 5 × 10⁶. Similar experiments with the Fg-Fab(E)-Fab’ polymer resulted in dissociation of the complexes during chromatography. Attempts to estimate the macro-molecular size of the Fg-Fab(E)-Fab’ and Fg-Fab(E) polymers by nonreduced SDS-PAGE on 3% gels resulted in dissociation of the polymer. Nondenaturing electrophoresis on hybrid 0.5%-3% acrylamide gels revealed failure of the fibrinogen polymers to penetrate even these large pore gels.

Platelet preparation. Blood was obtained by atraumatic venipuncture from healthy volunteer donors who denied having taken aspirin or other drugs affecting platelet function during the preceding 2 weeks and was anticoagulated with 3.8% sodium citrate (9:1). After centrifugation (2,000 g, 2 min) prostaglandin E, (PGE,) was added to 20 mL of platelet-rich plasma (PRP) and the platelets were pelleted by centrifugation (425 g, 15 min, 20 °C). The pellet was resuspended in 1 mL HEPES-Tyrodes buffer (129 mmol/L sodium chloride, 8.9 mmol/L sodium bicarbonate, 2.8 mmol/L potassium chloride, 0.8 mmol/L magnesium chloride, 0.8 mmol/L L-magnesium chloride, 0.8 mmol/L potassium phosphate, 0.8 mmol/L magnesium chloride, 2.4 mmol/L calcium chloride, 10.9 mmol/L sodium citrate, 5.6 mmol/L glucose, 10 mmol/L HEPES, and 0.35% bovine serum albumin), to which PGE, had been added. The resuspended platelets were then washed by passage over a 12 mL Sepharose 2B column preequilibrated with HEPES-Tyrodes, and the gel-filtered platelets (GFP) were diluted with HEPES-Tyrodes to a count of 5 × 10⁶. Aequorin-loaded platelets (ALP) were prepared as described by Johnson et al and measurements of ionized cytoplasmic Ca²⁺ by aequorin luminescence were conducted as described.

Aggregation of platelets. The effect of the polymer preparations on stirred suspensions of gel-filtered platelets was tested at 37 °C in an aggregometer. Control samples of GFP to which 500 µg/mL fibrinogen followed by 5 µmol/L ADP were added, aggregated normally (Figs 1 and 2). Addition of fibrinogen alone (Fig 2) induced no change in light transmission, suggesting that the platelets were not responsive to agonists. The murine monoclonal antibody 10E5, which binds specifically to the platelet membrane glycoproteins IIb/IIIa, was purified from ascites fluid by affinity chromatography on Protein-A Sepharose as described by Ey et al. In some experiments aspirin was added to GFP and incubated for 30 minutes at 22 °C.

RESULTS

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Addition of Fg-DMS (50 µg/mL) to GFP did not produce platelet aggregation or shape change, nor did it substitute for fibrinogen alone (Fig 2). Addition of ADP (5 µmol/L) alone induced shape change, as indicated by a fall in light transmission, but did not produce platelet aggregation, suggesting that the GFP did not contain significant residual fibrinogen in the medium.

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fibrinogen in support of platelet aggregation after addition of ADP (5 μmol/L) to the stirred mixture (Fig 2). Addition of Fg-DMS, then fibrinogen, and then ADP (5 μmol/L) to GFP did not reveal any inhibition of aggregation by the Fg-DMS polymer, and was not different from aggregation of platelets by ADP (5 μmol/L) in the presence of fibrinogen (500 μg/mL). Results obtained with Fg-F13 (20 μg/mL) were essentially the same as for Fg-DMS (data not shown). Thus, neither Fg-DMS nor Fg-F13 stimulated platelet aggregation, and neither supported or inhibited ADP-induced aggregation.

In contrast, addition of Fg-Fab(E) over a range of 35 μg/mL to 280 μg/mL aggregated GFP in a dose-dependent fashion (Fig 1, 3). Addition of exogenous ADP was not required for this response. Addition of anti-E-fragment Fab alone at concentrations up to 300 μg/mL did not produce platelet aggregation. Additions of fibrinogen alone at concentrations up to 500 μg/mL also produced no detectable aggregation. Addition of Fg-Fab(E) (140 μg/mL) to citrated platelet-rich plasma also caused platelet aggregation (data not shown).

Platelet aggregation in response to the three component (Fg-Fab(E)-Fab) polymer was also studied. When Fg-Fab(E)-Fab polymer was added to stirred GFP at 37°C there was a small increase in light transmission (data not shown). Phase microscopic examination of samples from the aggregometer cuvette showed many small platelet aggregates (estimated to contain 3 to 10 platelets) in samples containing Fg-Fab(E)-Fab, but not in the controls. These aggregates were quantified by measuring the number of residual “singlet” platelets by the method of Kohanna et al. In this method, doublets are counted as single platelets. After exposure to Fg-Fab(E)-Fab, 70% of platelets were measured in the “singlet” window, compared with 96% of those exposed to fibrinogen alone and 100% of those exposed to the Fab against fragment E alone.
not with fibrinogen and monovalent Fab specific for the E fragment. Neither fibrinogen nor Fab$_2$(E) alone aggregated platelets. These results suggest that interaction of fibrinogen with Fab$_2$(E) (i.e., polymer formation) was necessary for platelet aggregation in these experiments.

*Effect of inhibitors of platelet aggregation.* To explore the possibility that the clumping of platelets observed after addition of fibrinogen polymer was due to passive platelet agglutination, GFP were incubated for 30 minutes at 20°C with metabolic inhibitors sodium azide (10 mmol/L) and 2-deoxyglucose (10 mmol/L), which have been shown to inhibit platelet metabolic processes.\(^{61,62}\) Under these conditions GFP failed to aggregate after addition of Fg-Fab$_2$(E) polymer (35–280 μg/mL) (Fig 5). They also showed no shape change or aggregation after addition of fibrinogen (500 μg/mL) and ADP (5 μmol/L). A simultaneous control sample of GFP (no inhibitor, incubated 30 minutes at 20°C) aggregated similarly to fresh GFP. These results suggested that aggregation of platelets by the Fg-Fab$_2$(E) polymer was an active, energy-requiring process that could not be attributed to agglutination alone.

We examined the possible role of ADP in the stimulation of platelets by the Fg-Fab$_2$(E) polymer. The covalent inhibitor of ADP, FSBA (100 μmol/L), in methanol was added to stirred GFP and incubated at 37°C for five minutes. Addition of fibrinogen (500 μg/mL) followed by ADP (10 μmol/L) to FSBA-treated GFP produced no aggregation. Fg-Fab$_2$(E)–induced aggregation was inhibited in this system (Fig 5). Methanol was without effect at the concentration employed. In other experiments the ADP scavenging system, phosphocreatine (CP, 2 mmol/L) and phosphocreatine kinase (CPK, 10 U/mL), was added to GFP stirred at 37°C. Addition of fibrinogen (500 μg/mL) and ADP (10 μmol/L) one minute after addition of CP/CPK produced only a small increase in light transmission, indicating virtually complete inhibition of aggregation. Addition of Fg-Fab$_2$(E) polymer to GFP in the presence of CP/CPK resulted in attenuation of aggregation. Thus, it would appear that, although the Fg-Fab$_2$(E) polymer can produce full-scale aggregation of GFP without addition of exogenous ADP, endogenous ADP plays a role in the aggregation response to the polymer.

Monovalent Fab fragments specific for the D domain of fibrinogen have previously been shown to inhibit fibrinogen binding to ADP-stimulated platelets and to inhibit aggregation of GFP in the presence of fibrinogen and ADP.\(^{65}\) We confirmed that anti-D–fragment Fabs inhibited aggregation of GFP by ADP (5 μmol/L) in the presence of fibrinogen (500 μg/mL) (anti-E–fragment Fabs were without effect; data not shown). When added to stirred GFP at 37°C these anti-D–fragment Fabs (100 μg/mL) completely inhibited aggregation induced by the Fg-Fab$_2$(E) polymer (Fig 5). This result indicated that aggregation of GFP induced by the Fg-Fab$_2$(E) polymer required accessibility of the D domain of fibrinogen, either on the polymer itself or on fibrinogen which might be released by the platelet in response to stimulation by the polymer.

Since the previous experiments suggested that interaction of the Fg-Fab$_2$(E) polymer with platelets involved the D domain of fibrinogen and therefore possibly fibrinogen’s conventional platelet binding site (identified as residing in the D domain at the carboxyl-terminal end of the gamma chain\(^{28}\)), we studied the role of the platelet membrane receptor for fibrinogen, glycoprotein IIb/IIIa complex, in the aggregation of GFP by Fg-Fab$_2$(E) polymer. The murine monoclonal antibody 10E5 was added (10 μg/mL) to platelets stirred at 37°C in the aggregometer (Fig 5). After a one minute incubation, addition of fibrinogen (500 μg/mL) and ADP (5 μmol/L) produced no aggregation. At the same concentration, the 10E5 antibody also prevented aggregation of GFP by Fg-Fab$_2$(E) polymer. This implies that the platelet membrane glycoprotein IIb/IIIa complex, target of the monoclonal antibody and presumed platelet fibrinogen receptor, is involved in the aggregation of GFP by the Fg-Fab$_2$(E) polymer.

Gel-filtered platelets that had been incubated with aspirin for 30 minutes at 20°C were also studied. The GFP failed to aggregate in response to arachidonic acid, and in response to fibrinogen (500 μg/mL) and ADP (5 μmol/L) they underwent primary aggregation but not secondary aggregation, as would be expected. Incubation of GFP with aspirin partially reduced the rate and extent of aggregation induced by the Fg-Fab$_2$(E) polymer (Fig 5).

*Release of serotonin.* The effect of Fg-Fab$_2$(E) polymer upon platelet secretion was tested with $^{14}$C-serotonin–loaded GFP in an aggregometer. As shown in Table 1, serotonin release induced by Fg-Fab$_2$(E) polymer was dose-dependent and paralleled the extent of aggregation. In contrast, addition of either fibrinogen or Fab$_2$ specific for the E fragment produced little release of serotonin. Metabolic inhibition with sodium azide and 2-deoxyglucose blocked release of serotonin in response to Fg-Fab$_2$(E). Inhibition of ADP with FSBA or CP/CPK produced substantial inhibition of the secretory response to Fg-Fab$_2$(E). Blockade of the IIb/IIIa
glycoprotein with the monoclonal antibody 10E5 prevented serotonin release, as did blockade of the D domain of the polymer with monovalent Fab specific for the D fragment. Aspirin pretreatment reduced the release of serotonin stimulated by Fg-Fab(E). These observations supported the conclusion that GFP exposed to Fg-Fab(E) polymer had not only become aggregated but that a transmembrane signal had been generated, producing internal activation that culminated in the release reaction.

**Measurement of cytoplasmic free calcium concentration.** With evidence that GFP exposed to the multivalent Fg-Fab(E) polymer had become activated internally, it was of interest to determine whether the platelet cytoplasmic calcium ion concentration was elevated in response to the polymer, as is the case with platelet agonists such as thrombin and ADP.39 Aequorin-loaded GFP were prepared and agonists were added to suspensions in a modified Lumaggregometer (Chronolog, Inc, Boulder, Co). Simultaneous recordings of aequorin luminescence and optical density, representing platelet aggregation, were obtained.42 As is seen in Fig 6, addition of the Fg-Fab(E) polymer resulted in a dose-dependent elevation of ionized calcium in the absence of exogenous ADP. Aggregation of aequorin-loaded platelets (ALP) followed the increase in calcium concentration. The rise in Ca²⁺ after addition of Fg-Fab(E) was abolished when the polymer was added in the presence of the monoclonal anti-IIIb/IIla antibody 10E5 (10 µg/mL). Addition of CP/CPK (sufficient to block aggregation of ALP by 5 µmol/L ADP in the presence of fibrinogen (500 µg/mL)) strongly attenuated the extent of aggregation but only slightly reduced the increase in [Ca²⁺]. Addition of FSBA, an inhibitor of ADP at the receptor level, inhibited aggregation and significantly reduced Ca²⁺ elevation. When Fg-Fab(E) was added to ALP that had been metabolically inhibited by incubation with Na azide (10 mmol/L) and 2-deoxyglucose (10 mmol/L), no increase in cytoplasmic [Ca²⁺] was observed. These data indicate that platelet cytoplasmic [Ca²⁺] is increased by Fg-Fab(E) by an energy-dependent mechanism, that this increase is a consequence of Fg-Fab(E) polymer interaction with the GP IIIb/IIla receptor, and that release of endogenous ADP enhances the response but is apparently not required for the rise in [Ca²⁺] to occur.

**DISCUSSION**

In these experiments we have prepared polymers of fibrinogen by several crosslinking techniques. Polymers prepared by methods that may alter the D-domain binding site failed to induce aggregation of GFP and failed to support GFP aggregation after stimulation with ADP. Polymers prepared by crosslinkage with DMS or activated Factor XIII did not support platelet aggregation after stimulation with ADP, probably because DMS reacts with lysine residues that are integral to the recognizability of the gamma chain locus for receptor binding,25-28 and because Factor XIII crosslinkage also affects this region.46 In contrast a polymer, Fg-Fab(E), prepared by a method that crosslinked fibrinogen domains thought to be unnecessary for platelet binding, was a sufficient and potent stimulus to aggregation of GFP. A related experiment was reported by Tollefson and Majerus7 who found that divalent anti-fibrinogen antibodies promoted secretion of platelets on which fibrinogen was adsorbed, but that monovalent antibody fragments to fibrinogen did not induce platelet activation. The cross-linkage of fibrinogen receptor complexes by divalent antibody could have produced platelet activation and aggregation similar to that seen in our results.

In our experiments platelet aggregation was blocked by a monovalent antibody fragment specific for the fibrinogen D fragment. These observations are consistent with localization
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of the primary fibrinogen binding site for platelets in the D region. The further observation of blockade of fibrinogen polymer-induced aggregation by the monoclonal antibody 10E5 (which has previously been shown to block fibrinogen binding to the ADP-induced platelet receptors for fibrinogen) suggests that the polymer may function by binding to the IIb/IIIa receptor and perhaps cause platelet activation by receptor crosslinkage or other mechanism. The aggregation by Fg-Fab(2)E was dose dependent and irreversible. The effect was attenuated by metabolic inhibitors, variably reduced by removal of ADP or blocking of ADP binding sites and partially inhibited by aspirin pretreatment. Initiation of effect was attenuated by metabolic inhibitors, variably reduced by removal of ADP or blocking of ADP binding sites and partially inhibited by aspirin pretreatment. Initiation of platelet aggregation by the polymer was associated with a marked rise in cytoplasmic calcium ion concentration, as measured by aequorin luminescence. Release of serotonin was induced by the polymer, and variable degrees of inhibition of serotonin release were seen after metabolic inhibition, blockade or enzymatic digestion of ADP, blockade of fibrinogen receptors with monoclonal antibody, and inhibition of cyclooxygenase activity with aspirin.

That the aggregation of GFP by Fg-Fab(2)E was attenuated by ADP scavenging systems or after incubation with FSBA suggests that ADP release from the platelet may mediate or facilitate the aggregation response to the polymer. The observation that no aggregation, serotonin release, or elevation of cytoplasmic free calcium ion was seen in response to fibrinogen alone is evidence against the possibility that these GFP were already subjected to ADP stimulation before exposure to the Fg-Fab(2)E polymer. Thus the polymer itself appears to be capable of stimulating the platelet initially, presumably by binding to fibrinogen receptors, without dependence on the receptor-inducing effects of either exogenous or platelet-derived ADP.

The increase in affinity of the platelet for fibrinogen after addition of ADP is thought to involve exposure of the platelet fibrinogen binding sites, perhaps via a change in conformation of the GP IIb/IIIa complex. Newman et al. have shown that glycoproteins IIb and IIIa are associated in a 1:1 stoichiometry in the plasma membrane of intact, nonactivated platelets. Collier has recently presented evidence that "unmasking" of the fibrinogen receptor binding site is the result of changes in the conformation or microenvironment of the IIb/IIIa complex. He observed that rates of binding of small antibody fragments (Fab(2) and Fab), prepared from a monoclonal antibody (7E3) specific for the IIb/IIIa receptor of unactivated platelets, were increased in proportion to their smaller size. These findings may be indications that fibrinogen is weakly associated with the resting GP IIb/IIIa receptor complex. However, according to our model, presentation of multiple fibrinogen reaction sites at high local concentration in native conformation may have sufficient multivalent affinity to lead to binding and activation despite the relative inaccessibility of the receptor binding site for fibrinogen in the resting platelet.

Polymers composed of repeating subunits, each of which expresses binding sites for cell membrane receptors, may behave quite differently in biological systems from the same number of unassociated subunits acting independently. The effects of "multivalency" of binding loci upon receptor affinity have been analyzed by Crothers and Metzger, who concluded that, as a general principle, multivalent ligands may have affinities for their receptors that are several orders of magnitude higher than corresponding monovalent ligands. This prediction has been verified experimentally by Hornick and Karush, who determined that divalent anti-DNP (2,4-dinitrophenol) antibodies have *K*ₐ of 2.9 × 10⁻¹² mol/L compared with 1.67 × 10⁻⁷ mol/L for the monovalent Fab fragment.

The mobile receptor hypothesis of Jacobs and Cuatrecasas states that membrane receptors may be freely diffusible laterally in the resting state but when bound by ligands may become associated into complexes that promote effector activities. Receptor concentration ("patching") stimulated by binding of multivalent ligands has been observed in several cell systems. Steric rearrangement of receptors has also been offered as an explanation for platelet behavior. Polley published elegant electron micrographs documenting the formation of clusters of GP IIb/IIIa complexes in response to platelet stimulation by thrombin. DeMarco et al. recently suggested that platelet interaction with type IIb von Willebrand factor may result in clustering of the IIb/IIIa receptor complex and facilitate platelet-fibrinogen binding and platelet aggregation. These observations are notably analogous to those we describe with the Fg-Fab(2)E polymer.

Although it is possible that platelet activation by fibrinogen polymers involves conformational alterations of the IIb/IIIa receptors, it is tempting to speculate that steric rearrangement of receptors in the platelet membrane may alter associations between the cell membrane and cytoskeletal elements, whether by generation of elevated cytoplasmic calcium ion concentration, production of diacylglycerol, or via some other signal, which in turn promotes the release of the contents of various cytoplasmic granules. Alternatively, interaction of fibrinogen polymers with the platelet might activate the cell by a cumulative effect on cytoplasmic calcium ion concentration. It is possible that multiple GP IIb/IIIa complexes, operating as calcium channels, might lead to an elevation of platelet-free [Ca²⁺] sufficient to activate the cell's internal excitatory processes.

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REFERENCES

37. Lindon JN, McManama GP, Kushner L, Merrill EW, Salzman EW: Does the conformation of adsorbed fibrinogen dictate platelet interactions with artificial surfaces. Blood (this issue)
PLATELET AGGREGATION BY FIBRINOGEN POLYMERS

63. Collier BS, Peerschke EI, Scudder LE, Sullivan CA: A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. J Clin Invest 72:325, 1983
73. Kahn CR, Baird KC, Jarrett DB, Flier JS: Demonstration that receptor cross-linking or aggregation is important in insulin action. Proc Natl Acad Sci USA 75:4209, 1978
Platelet aggregation by fibrinogen polymers crosslinked across the E domain

G McManama, JN Lindon, M Kloczewiak, MA Smith, JA Ware, J Hawiger, EW Merrill and EW Salzman