

# Fibronectin and the multiple interaction model for platelet-collagen adhesion

(aggregation/antibody/electrophoresis/gelatin)

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**ABSTRACT** A rapid, sensitive, and reproducible assay to determine the adhesion of platelets to collagen has been developed. Collagen fibers and adherent platelets are retained on polycarbonate membrane filters. Chemical modification of collagen by acetylation and of platelets by treatment with chymotrypsin markedly reduces adhesion. The role of fibronectin in the collagen-platelet interaction has been examined. Treatment of platelets with purified antibody or Fab' fragments to fibronectin only slightly reduces adhesion. Preincubation of platelets with high concentrations of gelatin reduces adhesion by only 22% but fails to inhibit aggregation. Thus, fibronectin has only a limited role in the adhesion of platelets to collagen and is either not involved in the adhesion that leads to aggregation or is only one of several adhesion mechanisms, any of which alone can initiate aggregation.

When the integrity of the vascular endothelium is destroyed, platelets rapidly aggregate at the site to form a hemostatic plug. Baumgartner (1) has demonstrated that fibrillar collagen is the most thrombogenic substance in vascular subendothelium. After first adhering to the collagen, the platelets undergo the release reaction in which ADP and other substances are released from secretory granules. In the presence of calcium these substances, especially ADP, result in formation of a platelet plug to close the vascular defect (2). The mechanism by which platelets adhere to collagen to initiate the hemostatic process is unknown.

Jamieson and colleagues (3) proposed that formation of an enzyme-substrate complex between a platelet surface collagen glucosyltransferase and galactosylhydroxylysine residues of collagen was the mechanism by which platelets adhere to collagen. Despite early support (4-6), studies in this (7, 8) and other laboratories (9, 10) indicate that this mechanism is unlikely.

It has been known for many years that the fibrillar form of collagen is required or, at least, is several orders of magnitude more effective in initiating aggregation than monomeric collagen (9, 11). Santoro and Cunningham (8) have recently proposed that adhesion of platelets to collagen and their subsequent aggregation result from the interaction of multiple binding sites on the platelet surface, which may exhibit only modest affinity in the monovalent state for some structural feature of the collagen molecule also multiply present in the collagen fibril. This concept is consistent with the similar capacities of the genetically distinct collagen types (8, 12, 13), including the ( $\alpha$ 1)<sub>3</sub> trimer (14), to initiate aggregation provided they are in a fibrillar form. This occurs despite the existence of regions of variation in amino acid sequence and different polypeptide chain compositions and may suggest that completely identical platelet binding sites are not present on the surface of these different collagen fibers. The inability of monomeric collagen to inhibit aggregation induced by the identical collagen in fibrillar form (8) further supports this model.

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Fibronectin, a collagen-binding protein with molecular weight of 440,000 composed of two 220,000-dalton polypeptide chains (15), is found in plasma and on the surface of various cells and has been implicated in the adhesion of fibroblasts to collagenous substrates (16, 17). Bensusan *et al.* (18) have recently identified fibronectin in urea extracts of washed platelets and as one of the several proteins remaining attached or adsorbed to collagen after disruption by sonication of platelets adherent to collagen. They have proposed that fibronectin may be the collagen receptor on platelets. Because fibronectin has been demonstrated to bind to most of the genetically distinct collagens (19), further investigation into its role as a platelet surface collagen-binding component is warranted.

In this paper we present a rapid assay for the determination of collagen-platelet adhesion and apply it to the examination of the possible role of fibronectin as the physiologic mediator of the collagen-platelet interaction.

## MATERIALS AND METHODS

**Collagen.** Rat skin acid-soluble collagen was prepared by the method of Bornstein and Piez (20). Reconstituted native-type fibrils were prepared by dialysis of solutions of collagen in 3% acetic acid against 0.02 M Na<sub>2</sub>HPO<sub>4</sub>. Gelatin was prepared by heating this collagen in a boiling water bath for 10 min. The preparation was allowed to cool to ambient temperature and used immediately. Rat skin insoluble collagen was prepared as described (21). It was modified with acetic anhydride according to the procedure of Fraenkel-Conrat (22).

**Antibodies.** Rabbit antiserum to human fibronectin was a gift from Deane Mosher (University of Wisconsin). A purified antibody to human fibronectin was generously provided by Rupert Timpl (Max Planck Institut, Martinsreid, West Germany). Both preparations were active against fibronectin as revealed by Ouchterlony double diffusion. Monovalent Fab' fragments were prepared from antiserum as described by Brackenbury *et al.* (23).

**Platelets.** Washed <sup>51</sup>Cr-labeled platelets were prepared as described by Brass *et al.* (24) except that phosphate replaced the Tris buffer. For use in the adhesion assay the platelets were suspended in Dulbecco's phosphate-buffered saline (25) supplemented with 5 mM glucose, 2 mM EDTA, and 0.3% bovine serum albumin. For aggregation studies EDTA was omitted and the solution was made 2 mM in CaCl<sub>2</sub>, 1 mM in MgCl<sub>2</sub>, and 0.05% in fibrinogen. Platelet counts were in the range of 2-2.5 × 10<sup>8</sup> per ml.

**Aggregation and Adhesion Assays.** Platelet aggregation was measured in a Chronolog aggregometer as described (8). Adhesion was measured by incubating 0.4 ml of <sup>51</sup>Cr-labeled platelets with 0.1 ml of a suspension (prepared by homogenization) of the desired quantity of reconstituted collagen fibrils or insoluble collagen at 37°C for 20 min in a reciprocal shaker operating at 100 cycles per min. The suspension was then ap-

plied to a 2.5-cm Unipore membrane (5- $\mu$ m pore size) obtained from Bio-Rad Laboratories mounted in a multiwell manifold that had been prerinsed with assay buffer. The membrane was then rinsed with 2 additional ml of assay buffer. Collagen fibrils and adherent platelets are retained on the membrane whereas nonadherent platelets pass through. The adherent platelets were then quantitated in a Beckman Gamma 4000 counter. The percentage of platelets in the incubation medium that adhered to collagen was determined.

**Chymotrypsin Treatment of Platelets.** Washed platelets were suspended at  $2.5 \times 10^8$  per ml in adhesion assay buffer that did not contain bovine serum albumin. The platelets were treated with 30  $\mu$ g of chymotrypsin per ml (three times crystallized; obtained from Worthington) at 37°C for the indicated time. The platelets were washed once by centrifugation and resuspended in adhesion assay buffer.

**Iodination of Platelets.** Washed platelets were labeled with  $^{125}$ I by lactoperoxidase-catalyzed iodination as described by Phillips and Agin (26).

**Polyacrylamide Gel Electrophoresis.** Electrophoresis on 5% polyacrylamide gels in the presence of sodium dodecyl sulfate was conducted as described by Weber and Osborn (27). Gels were sectioned into 1-mm slices and the radioactivity was measured in a Beckman Gamma 4000 counter.

## RESULTS

**The Adhesion Assay.** Preliminary studies revealed that the adhesion of platelets to collagen fibers as determined by this assay occurs in a time-dependent manner and is essentially complete in 10–15 min. Twenty minutes was, therefore, selected as the standard assay time. The extent of adhesion approximates a linear function of the amount of collagen present (Fig. 1). This suggests that under the conditions of the assay the amount of collagen present limits the extent of adhesion. Light and scanning electron microscopy revealed that no aggregation occurred and that only single platelets adhered to the collagen fibers in the EDTA-containing medium. Although the 5- $\mu$ m-pore-size membranes, in contrast to membranes of smaller pore size, did not appear to be clogged by collagen or platelets, it was necessary to rule out the possibility that the results obtained were due to trapping of platelets by a collagen mat on

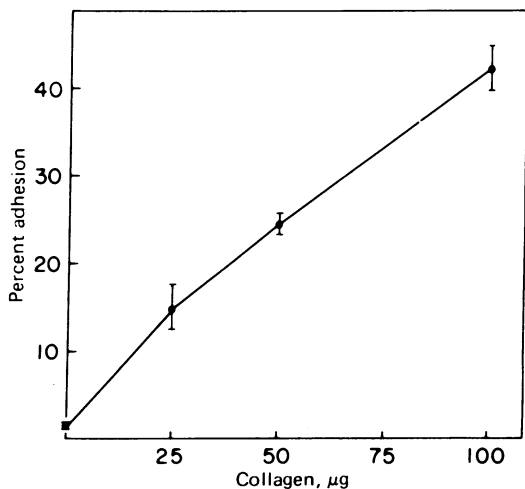


FIG. 1. Dependence of adhesion assay on concentration of collagen fibrils. Washed, labeled platelets (0.4 ml),  $2 \times 10^8$  per ml, were incubated with 0.1 ml of assay buffer containing the indicated amount of collagen fibrils. Adhesion is plotted as the percentage of total platelets adhering to collagen. Error bars represent the range of duplicate determinations.

the membrane. To test this possibility, platelets incubated with and without collagen were applied to the membranes. In addition, platelets incubated in the absence of collagen were applied to membranes to which 100  $\mu$ g of reconstituted collagen fibrils had been previously applied. The results (Fig. 2) clearly indicate that the observed adhesion is not due to trapping of platelets by collagen. The number of platelets retained on the membrane already containing collagen did not greatly differ from the number retained in the absence of collagen. Similar results were obtained by using 400  $\mu$ g of insoluble collagen. If insoluble collagen that had been briefly treated with acetic anhydride was used as substrate, adhesion was only 52% of the value observed with unmodified collagen. This result not only indicates that the adhesion assay is affected by collagen modification, but also implicates collagen amino groups in the adhesion of platelets to collagen.

**Effect of Chymotrypsin Treatment of Platelets.** The effect of platelet surface alteration by protease treatment was examined. Chymotrypsin was selected because it does not induce the release reaction and initiate aggregation as do other proteases such as trypsin and thrombin (28). Treatment of platelet suspensions with 30  $\mu$ g of chymotrypsin per ml decreased the extent of adhesion of platelets to collagen. The decrease was dependent upon the time of enzyme treatment (Fig. 3). Thus, the adhesion of platelets to collagen is likely mediated by platelet surface proteins.

To examine the platelet surface alterations resulting from the chymotrypsin treatment, iodinated platelets were treated with chymotrypsin and the enzyme-treated platelets were examined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 4). The surface changes were major and complex and it was not possible to correlate the decrease in adhesion with a decrement in any particular platelet surface protein. The region of the gel corresponding to fibronectin, molecular weight 220,000–250,000, represents only a minor portion of the labeled surface proteins of the intact platelet. This

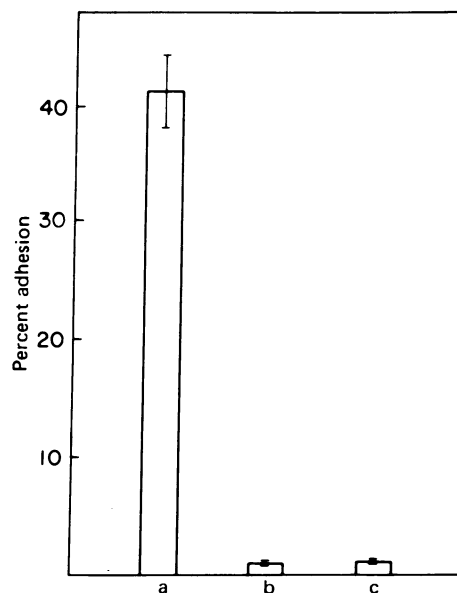


FIG. 2. Failure of collagen fibrils to trap nonadherent platelets. Washed, labeled platelets (0.4 ml),  $2.4 \times 10^8$  per ml, were incubated with 0.1 ml of assay buffer or assay buffer containing 100  $\mu$ g of fibrillar collagen. The retention of these platelets on membrane filters was then determined. Bars: a, Platelets incubated with 100  $\mu$ g fibrillar collagen; b, platelets incubated in the absence of collagen; c, platelets incubated in the absence of collagen but applied to membranes containing 100  $\mu$ g of fibrillar collagen.

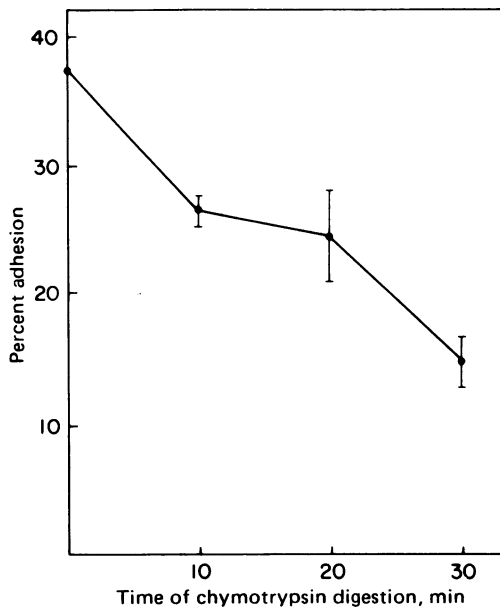


FIG. 3. Effect of chymotrypsin treatment on collagen-platelet adhesion. Washed, labeled platelets (2 ml),  $2.5 \times 10^8$  per ml, were treated with  $30 \mu\text{g}$  of chymotrypsin per ml for the indicated times. The platelets were washed once by centrifugation and resuspended in adhesion assay buffer. Buffer (0.1 ml) containing  $300 \mu\text{g}$  of insoluble collagen was added to duplicate 0.4-ml aliquots of the platelet suspension, and adhesion was determined.

is in marked contrast to the much larger proportion seen in fibroblasts (15). During the 30-min enzyme treatment, the radioactivity in this region of the gel was reduced to baseline values.

**Role of Fibronectin in Collagen-Platelet Adhesion.** Antibodies to cell surface components are effective inhibitors of both cell-cell (23, 29) and cell-substrate adhesion (30). If platelet surface fibronectin is responsible for the adhesion of platelets to collagen, antibodies directed against fibronectin may inhibit the adhesion of platelets to collagen. Preincubation of platelets for 30 min with up to  $41 \mu\text{g}$  of purified antibody per ml to fibronectin resulted in only a 24% inhibition of adhesion (Fig. 5). Microscopic examination of the antibody-treated platelets revealed no agglutination. However, after centrifugation to remove unbound antibody, the antibody-treated platelets, in contrast to control platelets, could not be readily resuspended. Therefore, the results in Fig. 5 were obtained by preincubating platelets with antibody and allowing the excess antibody to remain present during the adhesion assay.

Because of the potential complications in interpreting data obtained with intact antibody, the effects on adhesion of monovalent Fab' fragments prepared from preimmune and antifibronectin rabbit sera were also examined. The adhesion in the presence of antifibronectin Fab' fragments was 90% of that obtained with control Fab' fragments. The same extent of inhibition was obtained over a 10-fold range of concentration of Fab' fragments.

The affinity of fibronectin for denatured collagen is significantly greater than for native collagen (19). If platelets are preincubated with high concentrations of denatured collagen and the adhesion assay is conducted in the presence of the denatured collagen, the platelet surface fibronectin should be saturated and unavailable to interact with fibrils of native collagen for which fibronectin exhibits a lower affinity. Preincubation of platelets for 30 min at  $37^\circ\text{C}$  with 1.5 mg of heat-denatured collagen per ml and determination of adhesion

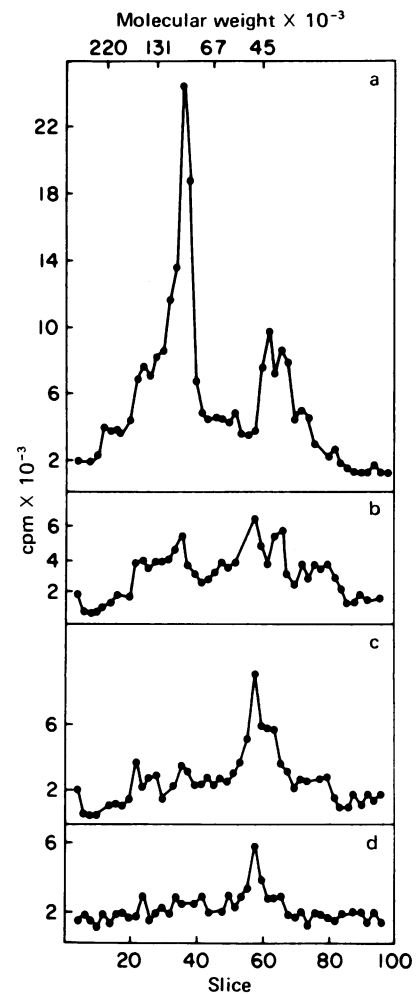


FIG. 4. Effect of chymotrypsin treatment on platelet surface proteins. Iodinated platelets were treated with chymotrypsin under the same conditions as Fig. 3. The washed enzyme-treated platelets were dissolved in 2% sodium dodecyl sulfate and examined on 5% polyacrylamide gels. The gels were sliced into 1-mm segments, and the radioactivity in each was determined. (a) Iodinated platelets not exposed to chymotrypsin. (b) Ten-minute chymotrypsin treatment. (c) Twenty-minute treatment. (d) Thirty-minute treatment.

in the presence of this concentration of gelatin resulted in only a 22% inhibition of adhesion. As shown in Fig. 6, as little as 0.2 mg of gelatin per ml resulted in the same extent of inhibition. Furthermore, gelatin alone failed to retain platelets on the membrane.

Adhesion between two large and complex surfaces such as platelets and collagen fibrils may not reflect a single, specific physiologic process but may be the result of interactions of widely varying nature and specificity. Aggregation is the physiologic consequence of, presumably, one specific mechanism of adhesion of platelets to collagen. If the small inhibition of adhesion produced by gelatin reflects inhibition of the physiologic process, then preincubation of platelets with gelatin should inhibit aggregation. That such is not the case is shown in Fig. 7. Preincubation of platelets with 1 mg of gelatin per ml did not inhibit aggregation induced by  $20 \mu\text{g}$  of reconstituted collagen fibrils. This concentration of gelatin also failed to alter the aggregation produced by a lower, suboptimal concentration of collagen. As reported (7, 29) gelatin alone was incapable of initiating aggregation.

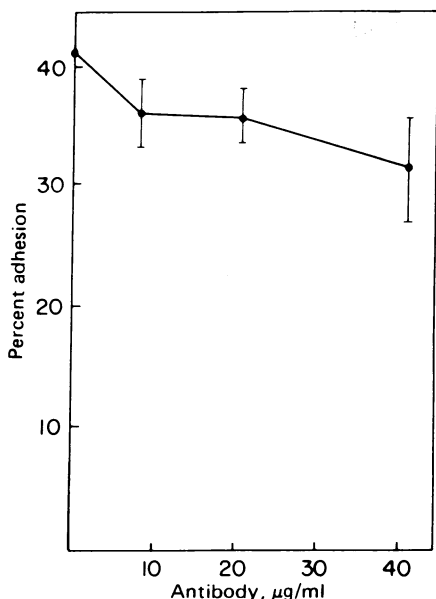


FIG. 5. Effect of purified antibody to fibronectin on collagen-platelet adhesion. Washed labeled platelets (0.4 ml),  $3 \times 10^8$  per ml, suspended in adhesion assay buffer, were incubated for 30 min at room temperature with 50 µl of assay buffer containing sufficient antibody to give the indicated concentration. Buffer (0.1 ml) containing 100 µg of fibrillar collagen was then added and adhesion was determined. Preimmune antiserum had no effect on adhesion.

DISCUSSION

Despite much investigation the biochemical nature of the collagen-platelet interaction remains unknown. This may be due in part to the unavailability of a rapid and sensitive adhesion assay suitable for the multiple determinations required for

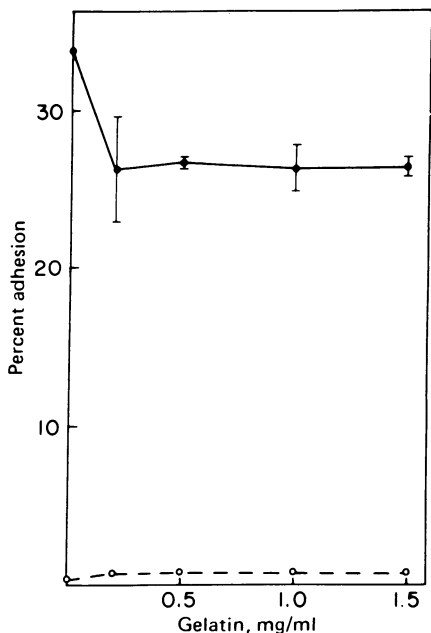


FIG. 6. Effect of denatured collagen on adhesion of platelets to fibrillar collagen. Platelets (0.4 ml),  $2.5 \times 10^8$  per ml, suspended in adhesion assay buffer containing the indicated gelatin concentration, were preincubated for 30 min at 37°C. Buffer (0.1 ml) or buffer containing 200 µg of fibrillar collagen (0.1 ml) was added, and adhesion was determined. ○, Gelatin-treated platelets retained on the membrane in the absence of fibrillar collagen; ●, gelatin-treated platelets retained on the membrane after exposure to 200 µg of fibrillar collagen.

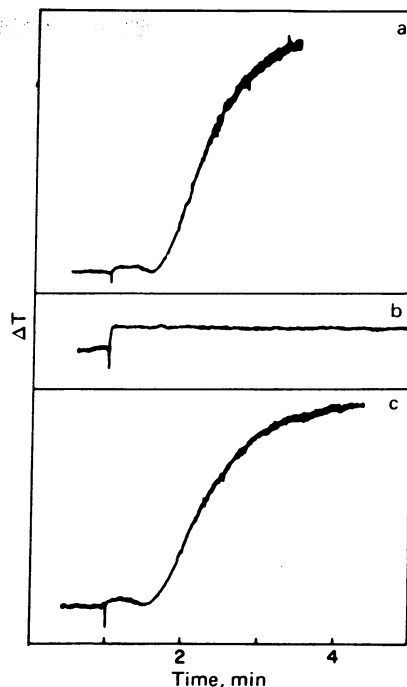


FIG. 7. Effect of denatured collagen on the aggregation of platelets by fibrillar collagen. Platelets were resuspended in aggregation assay buffer at  $2.5 \times 10^8$  per ml and aggregation was measured as described (8). (a) Fibrillar collagen (20 µg). (b) Denatured collagen (500 µg). (c) Platelets were incubated in the aggregometer for 5 min with 500 µg of denatured collagen and then 20 µg of fibrillar collagen was added.

biochemical investigation. From the data in Fig. 1, one can calculate that approximately 320,000 platelets adhere to each µg of collagen fibers. This number actually conveys little information, however, because adhesion is dependent upon the exposed surface area, not upon the mass of collagen. Preparations of fine fibrils are more effective per unit mass. Furthermore, this number reflects only a lower limit due to the vigorous shaking and washing procedures employed and thus represents only tightly adhered platelets.

The assay described here is sufficiently sensitive to reflect the effect of chemical or enzymatic alterations of either collagen or the platelet surface. Initial experiments have implicated the collagen amino groups and platelet surface proteins in the collagen-platelet interaction. Collagen amino groups have been shown to be important in collagen-induced aggregation (31). We have extended these observations to adhesion. Use of insoluble collagen rather than reconstituted fibrils in this particular comparison was necessary to circumvent potential problems with respect to fibril formation by acetylated soluble collagen.

The adhesion assay has been used to examine the role of platelet surface fibronectin in collagen-platelet adhesion as recently proposed by Bensusan *et al.* (18) in light of our proposal concerning the role of multivalent interactions (8). Treatment of platelets with purified antibody to fibronectin results in only 25% inhibition of adhesion. Fab' fragments result in only 10% inhibition. Because fibronectin has a higher affinity for gelatin than for native collagen (19), preincubation of platelets with high concentrations of gelatin should inhibit the adhesion of platelets to collagen. A similar approach resulted in marked inhibition of the adhesion of fibroblasts to a collagen substrate (32). With platelets, only a 20% inhibition was obtained with high gelatin concentrations. It thus appears that platelet surface fibronectin, whether it represents adsorbed plasma cold insol-

uble globulin or the cell surface form of fibronectin, can account for only 10–20% of the observed adhesion to collagen. That this decrement in adhesion does not affect the physiologic consequence of adhesion, platelet aggregation, is reflected in the inability of gelatin to produce any inhibition in collagen-induced aggregation even at suboptimum collagen concentrations.

The adhesion of platelets to collagen may be mediated by several different mechanisms. Clearly, any fibronectin on the platelet surface, whether it is adsorbed plasma fibronectin or a true peripheral component of the platelet surface, will contribute to the adhesion of platelets to collagen. Either fibronectin is not involved in adhesion leading to aggregation, or it is only one of several adhesion mechanisms, any of which alone can initiate release and aggregation.

The requirement for fibrillar collagen to initiate aggregation (9, 11), the inability of monomeric collagen to inhibit aggregation (8), and the inability of gelatin to inhibit aggregation or adhesion all suggest that interaction of platelets with the matrix formed by fibrillar collagen plays a key role in the initiation of aggregation by collagen. The multivalent interaction model also predicts that the solubilization and dispersal of the collagen receptors into "monovalent" form could produce a molecule with only moderate affinity for collagen. This could account for the various platelet proteins, including a number of platelet surface components, isolated by affinity procedures by us (unpublished observations) and others (16). The platelet receptor(s) responsible for adhesion related to the physiological event of aggregation thus continue to resist identification, but fibronectin clearly plays at best a limited role.

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