

# Identification and Quantitation of Platelet-Associated Fibronectin Antigen

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**ABSTRACT** Platelet-associated fibronectin antigen has been identified by radioimmunoassay and immunofluorescent techniques. In radioimmunoassay, platelet fibronectin was immunochemically indistinguishable from plasma fibronectin. Platelet and plasma fibronectin were bound and eluted from gelatin-sepharose under similar conditions. The level of platelet fibronectin in detergent extracts of washed platelets from 12 healthy adults was  $2.85 \pm 1.24 \mu\text{g}/10^9$  platelets. Immunofluorescence with  $\text{F}(\text{ab}')_2$  fragments of immunochemically purified antifibronectin showed that all platelets stained with a discrete punctate pattern. The identification of platelet fibronectin antigen raises the possibility that this protein may participate in platelet-platelet or platelet-surface interactions.

## INTRODUCTION

Platelet adhesion and aggregation are fundamental events in the hemostatic process. The fibronectins (fn),<sup>1</sup> such as cold insoluble globulin (1) and the large external transformation-sensitive protein (2), are a family of widely distributed glycoproteins found in plasma and in association with the surfaces of cells, including fibroblasts and endothelial cells (3, 4). This protein was originally described by Morrison et al.

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<sup>1</sup>Abbreviations used in this paper: fn, fibronectin(s); PBS, 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.3; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

(1) in 1948 and has now been implicated in a wide variety of cellular activities, which primarily involve adhesion of cells to one another or to substrata. The participation of fn in cell agglutination, aggregation, and adhesion reactions has been demonstrated in a number of studies (3, 4). The presence of fn within the platelet was suggested by Mosesson and Umfleet (5) in 1970 but was not rigorously documented and has not been confirmed. With the increasing evidence for the role of fn in cell-cell interactions and the basic importance of such interactions in platelet hemostatic function, we have sought to verify the presence and quantify the level of fn antigen in platelets.

## METHODS

**Plasma fn.** Plasma fn was purified by affinity chromatography on gelatin-Sepharose (Pharmacia Fine Chemicals, Piscataway, N. J.) as described by Engvall and Ruoslahti (6) with 2 ml plasma/ml beads. Bound fn was eluted with either 6 M urea, pH 7.5 or 1 M NaBr, pH 5.3. The latter eluates were dialyzed against 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.3 (PBS) and used for radioiodination.

**Anti-fn.** Anti-fn was prepared by immunization of rabbits or goats with 1-mg subcutaneous doses in complete Freund's adjuvant on a bimonthly schedule. The anti-fn used in this study was absorbed by passage through a gelatin-Sepharose column and a Sepharose column to which plasma depleted of fn was coupled. Fn antibodies were immunochemically purified on fn-Sepharose (5 mg fn/ml beads). After application of the antiserum and thorough washing, elution with 3.0 M KI in PBS yielded one major component of 160,000 mol wt on nonreduced polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE).  $\text{F}(\text{ab}')_2$  fragments were prepared by digestion of 7.5 mg purified antibody with 200  $\mu\text{g}$  of pepsin at pH 4.0 for 18 h at 37°C followed by extensive dialysis. Control  $\text{F}(\text{ab}')_2$  fragments were prepared from the IgG fraction isolated by DEAE-cellulose chromatography of pre-immunization bleedings. Digestion of >97% of the IgG to  $\text{F}(\text{ab}')_2$  fragments was estimated from SDS-PAGE.

**Radioimmunoassay.** Fn was radiolabeled with <sup>125</sup>I to a specific activity of 0.5–1  $\mu\text{Ci}/\mu\text{g}$ , and the double antibody radioimmunoassay was similar to assays previously described by this laboratory (7). Assays were performed in

plastic, siliconized tubes at 22°C with  $^{125}\text{I}$ -fn at a final concentration of 15 ng/ml in a buffer system of 0.025 M NaCl, 0.04 M sodium borate, pH 8.3 containing 1% heat-inactivated normal rabbit serum and 1 mM EDTA. The precipitability of the ligand in 10% trichloroacetic acid was routinely >90% and nonspecific precipitation by second antibody was <5%.

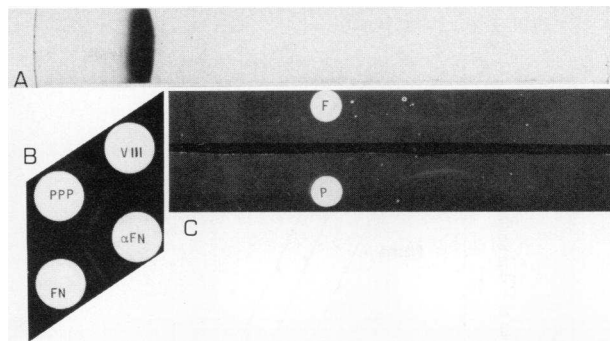
**Platelets.** Platelet-rich plasma was prepared as described (8) and the platelets were pelleted by centrifugation at 1,000 g for 20 min and resuspended in modified Tyrode's buffer (8). The suspension was then gel filtered (9) on Sepharose 2B-CL in modified Tyrode's. The washed platelets were counted electronically and exhibited typical aggregation and secretion responses to thrombin, ADP, and collagen.  $^{125}\text{I}$ -fn, added to the platelet-rich plasma was not detected in the isolated platelet fraction, and, on this basis, a contribution of  $\leq 200$  ng fn/ $10^9$  platelets caused by plasma contamination was calculated. For extraction, platelets were pelleted at 1,000 g for 10 min and 0.5% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) added. After 30 min at 22°C, the supernate was retrieved by centrifugation for analysis. When isolated plasma fn was added to platelet extracts, the fn antigen was quantitatively recovered in the supernatant fraction after an 18-h incubation, indicating stability of the antigen in the extracts.

**Immunofluorescence.** Immunofluorescent staining was performed on 2% formaldehyde fixed platelets on polylysine-coated, circular glass cover slips. The cells were treated for 3 min with 0.1% Triton X-100 to render them permeable to antibody and incubated for 20 min with either goat F(ab')<sub>2</sub>, anti-fn, or nonimmune F(ab')<sub>2</sub>. The cells were rinsed with PBS and stained for 20 min with rhodamine-labeled rabbit F(ab')<sub>2</sub> antigoc goat immunoglobulin (N. L. Cappel Laboratories Inc., Cochranville, Pa.). The platelets were viewed with a Zeiss universal microscope equipped with an HBO SOW mercury lamp and an IVFI epifluorescence condenser with a BP 546 excitation filter, a KT 580 chromatic splitter, and a LP 590 barrier filter (Carl Zeiss, Inc., New York).

**SDS-PAGE.** SDS-PAGE (10) was performed in 5% gels in the presence or absence of 1% 2-mercaptoethanol. Molecular weights were estimated from the mobility relative to protein standards.

## RESULTS

The characteristics of isolated plasma fn and the antisera used in this study are shown in Fig. 1. Isolated fn, when reduced with 1% 2-mercaptoethanol, revealed only a single major component on 5% SDS-PAGE with an estimated mol wt of 230,000 (Fig. 1A). This apparent molecular weight of the fn subunit(s) is consistent with the reported values (11, 12). When radiolabeled, the reduced  $^{125}\text{I}$ -fn also migrated as a single component with a relative mobility identical to the unlabeled fn subunit, and >85% of the radioactivity was recovered in the single major peak. In immunodiffusion analysis (Fig. 1B), goat anti-fn formed a single precipitin arc with normal plasma and yielded a reaction of complete identity with isolated fn. Factor VIII antigen did not form a precipitin arc with the antiserum and produced no apparent deviation of the precipitin formed with fn. In immunoelectrophoresis (Fig. 1C), anti-fn produced a single precipitin



**FIGURE 1** Characterization of isolated plasma fibronectin and its antiserum. (A) SDS-PAGE of isolated fn. A 25- $\mu\text{g}$  sample was reduced with 1% 2-mercaptoethanol and electrophoresis was performed on a 5% polyacrylamide gel. The gel was stained with Coomassie brilliant blue. (B) The double immunodiffusion analysis was performed in 1% agarose in barbital buffer, pH 8.6. In the wells were 12.5  $\mu\text{l}$  of isolated plasma fn (300  $\mu\text{g}/\text{ml}$ ), platelet-poor plasma (PPP), Factor VIII antigen (1 U/ml), and goat anti-fn. (C) Immunoelectrophoresis was performed in 1% agarose in veronal buffer, pH 8.6 at 5 V/cm for 60 min with 3  $\mu\text{g}$  fn and 10  $\mu\text{l}$  plasma/well, and precipitins were developed with 100  $\mu\text{l}$  of goat anti-fn.

arc with plasma in a position identical to purified fn. The rabbit anti-fn used exhibited similar specificity, and in radioimmunoassay showed no binding of  $^{125}\text{I}$ -fibrinogen.

A radioimmunoassay of the double antibody type was developed to detect fn antigen in washed platelets, with isolated plasma fn as a standard for quantitation. When platelets from solutions that contained from 0.5 to  $2 \times 10^9$  platelets/ml, isolated by differential centrifugation and gel filtration, were pelleted by centrifugation,  $\leq 50$  ng/ml fn was detected in the supernates indicating minimal carryover of soluble plasma fn. This is consistent with the failure to detect significant contamination of  $^{125}\text{I}$ -fn added to platelet-rich plasma. In contrast, fn antigen was detected in the 0.5% Triton extract of the washed platelets. (Triton alone was without effect on the assay). As shown in Fig. 2, the platelet lysate produced complete competitive inhibition as did plasma fn and plasma indicating apparent antigenic identity between the three samples. Thus, with this antiserum, platelet fn was essentially indistinguishable from plasma fn. Analysis of the platelet extract from 12 adult donors yielded an average fn level of  $2.85 \pm 1.24$  (SD)  $\mu\text{g}/10^9$  platelets. The range was from 1.24 to 7.20  $\mu\text{g}/10^9$  platelets. A plasma fn level of  $270 \pm 176$   $\mu\text{g}/\text{ml}$  was obtained from eight donors, consistent with reported levels (5). When platelet extracts were chromatographed on gelatin-Sepharose, fn antigen was bound and recovered in the 1 M NaBr eluate. Thus, the platelet fn antigen was immunochemically and functionally similar to plasma fn.

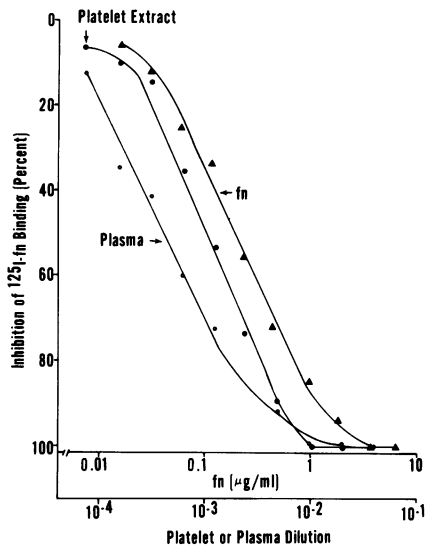


FIGURE 2 Competitive inhibition radioimmunoassay for fn antigen. Competing antigens were isolated plasma fn (▲), normal plasma (○), and the Triton X-100 extract of  $3 \times 10^9$  platelets (●). The  $^{125}\text{I}$ -fn was present at 15 ng/ml and the 1:800 dilution of rabbit anti-fn used bound 40% of the ligand.

The above experiments established that the detected fn antigen was cell associated. To determine whether the antigen was platelet derived, indirect immunofluorescent staining of the cell suspension was performed with the  $\text{F}(\text{ab}')_2$  fragments of immunochemically purified goat anti-fn and rhodamine-labeled  $\text{F}(\text{ab}')_2$  fragments of rabbit anti-goat IgG. As shown in

Fig. 3, all platelets stained with the anti-fn yielded a punctate pattern with multiple discrete fluorescent foci per cell. Addition of fn to the antiserum before staining completely blocked cell staining, producing a pattern similar to that obtained with pre-immune  $\text{F}(\text{ab}')_2$  fragments.

## DISCUSSION

In this study, an antigen related to plasma fn has been identified in platelets. In radioimmunoassay, the plasma and platelet fn antigens were immunochemically indistinguishable, consistent with previous reports (3-5) of the immunochemical similarity of plasma and cell-associated fn. In addition, the functional property of fn to bind to gelatin was also exhibited by the platelet fn antigen. Immunofluorescence established the identity of the fn-bearing cell as a platelet and further demonstrated that most platelets carry the antigen. The platelet fn antigen identified was not attributable to plasma fn contamination based upon the failure to detect  $^{125}\text{I}$ -fn, added to the platelet-rich plasma in the platelet fraction, or fn antigen in the isolated platelets before cell lysis. A number of plasma molecules including fibrinogen, Factor XIII, and Factor VIII antigen have been identified as intracellular constituents of the platelet (13). Platelets also bear receptors for molecules such as thrombin (14) and Factor Xa (15). The present study does not permit us to distinguish between an intracellular or a membrane-associated form of fn.

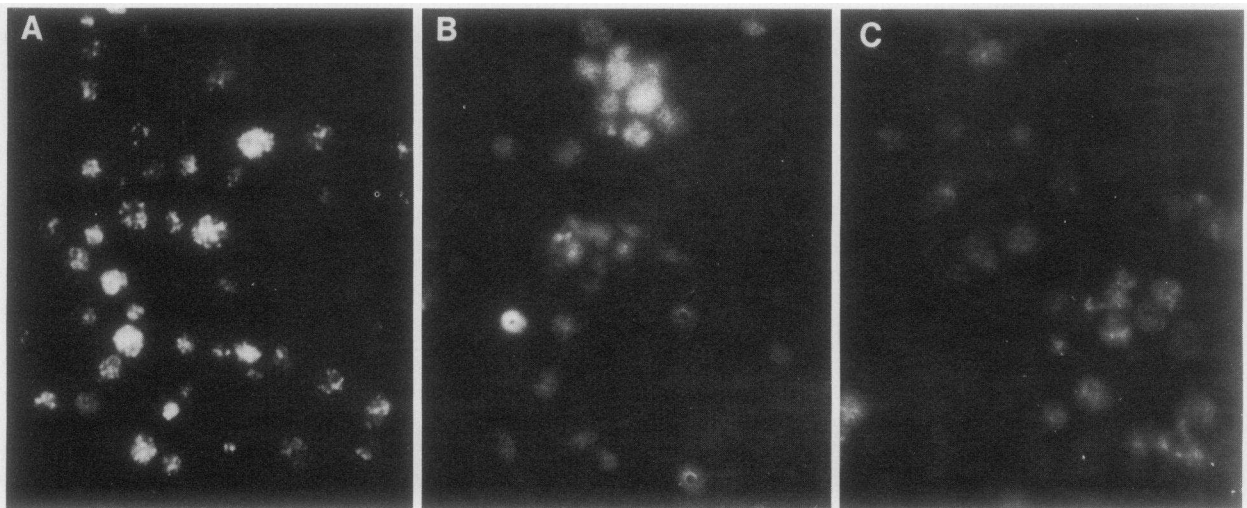


FIGURE 3 (A) Immunofluorescent staining of formaldehyde fixed and 0.1% Triton X-100 treated platelets by  $\text{F}(\text{ab}')_2$  fragments (50  $\mu\text{g}/\text{ml}$ ) of immunochemically purified goat antiserum to fn. The fluorescence was developed with rhodamine conjugated  $\text{F}(\text{ab}')_2$  fragments of rabbit antiserum to goat IgG. (B) Blocking control in which an equal volume of  $\text{F}(\text{ab}')_2$  fragments (100  $\mu\text{g}/\text{ml}$ ) of the anti-fn was incubated with isolated plasma fn (300  $\mu\text{g}/\text{ml}$ ) for 20 min before staining. (C) Pattern obtained with the rhodaminated antibody in the absence of anti-fn.

The detection of platelet fn raises the possibility that certain platelet interactions may be mediated by this protein. Platelets adhere to collagen and fibrin(ogen) with induction or potentiation of platelet aggregation and release (16) and these molecules also bind to fn (3, 4, 6). Factor XIII is present in platelets and is capable of cross-linking fn to itself or to fibrin (12). Interaction of these molecules with platelet fn either directly or after platelet stimulation might contribute to platelet-platelet and platelet-surface interactions.

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