

Specific Binding of Fibronectin–Antifibronectin Immune Complexes to Procollagen: A New Pitfall in Immunostaining

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ABSTRACT We observed intense intracellular immunofluorescence of rat lung fibroblasts stained with hybridoma culture supernatant containing monoclonal antibodies to human plasma fibronectin, but no pericellular matrix staining. Immunoprecipitation and absorption experiments revealed that this intracellular staining by hybridoma-conditioned medium was due to binding of fibronectin-antifibronectin immune complexes via the fibronectin to intracellular procollagen. The anomalous staining patterns we encountered were not revealed by the usual controls for immunohistochemical specificity, and also occurred in rat tissue sections. This general phenomena—binding of serum antigens present in hybridoma medium to cellular components—could in principle result in artifactual staining with monoclonal antibodies to other serum components, so investigators using monoclonal antibodies should be aware of this new artifact. Our results also demonstrate that fibronectin binds specifically to native procollagen. Monoclonal antibodies may be useful for studying fibronectin-procollagen and other macromolecular interactions.

Monoclonal antibodies (7) are powerful tools for cell biologists, but their use may be accompanied by unusual pitfalls. For example, it is well documented that monoclonals may bind genetically distinct polypeptides (8). Using hybridoma culture medium containing monoclonal antibody to human fibronectin (FN)¹ to stain rat lung fibroblasts (RLF), we encountered a previously unreported artifact. Monoclonal antifibronectin-fibronectin complexes bound to intracellular collagenase-sensitive ligands, presumably procollagen, resulting in intense intracellular immunofluorescence. As monoclonals are often used in the form of conditioned medium from serum-containing hybridoma cultures, this general phenomena is important to recognize as a potential artifact of monoclonal antibody staining. Our findings also have implications for fibronectin interactions with procollagen, which are briefly discussed.

MATERIALS AND METHODS

Immunoreagents and FN: Monoclonal antibodies to human

¹ *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; FN, fibronectin; RLF, rat lung fibroblasts.

plasma FN were obtained by fusing spleen cells from immunized BALB/c mice with SP2/O-Ag 14 myeloma cells (19). Positive fusions were cloned in soft agar and antibody-secreting clones detected by enzyme-linked immunosorbent assay (4). Hybridomas were expanded in cell culture using DME plus 10% agammaglobulinemic horse serum, 5% calf serum, and nonessential amino acids ("hybridoma medium"). Ascites fluid containing 2–5 mg/ml of IgG was obtained by intraperitoneal injection of 10⁶ myeloma cells into pristane primed BALB/c mice followed by paracentesis after 1–2 wk. Ascites was clotted at 25°C for 1 h, centrifuged (1,000 g, 10 min), protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, 10 mM EDTA) and 0.02% NaN₃ were added, and the supernate ("ascites") stored at –20°C. In some experiments IgG monoclonals were purified by affinity chromatography on FN-Sepharose. Human plasma FN, rat plasma FN, and hybridoma medium FN (from the horse and calf serum) were purified by gelatin-affinity chromatography (11). For maximal purity, the human plasma FN was further purified on an IgG monoclonal antibody to human plasma FN coupled to Sepharose-4B. For absorption experiments, conditioned hybridoma medium or ascites fluid (1/100 dilution) was incubated with 1 mg/ml purified FN for 2 h at 37°C, centrifuged (10,000 g, 5 min) and the supernatant used for staining. Polyclonal rabbit anti-human plasma FN IgG was affinity-purified as described (12, 20). FN absorption of polyclonal anti-FN was performed as above, with a 5:1 molar ratio of FN/IgG, using 440 kilodalton (kd) and 150 kd for FN and IgG.

Cell Culture, Metabolic Labeling and Immunoprecipitation: Rat lung fibroblasts were obtained from trypsin digests of minced lungs of 21-d Sprague-Dawley rat embryos and maintained in RPMI-1640 containing 25 µg/ml ascorbic acid and 10% fetal bovine serum depleted of

gelatin-binding activity by absorption on gelatin-Sepharose (13). RLF were used within five subcultivations (1:3 split ratio) and maintained typical fibroblastic morphology. These cells stained uniformly for intracellular and matrix FN with polyclonal anti-FN IgG (see below).

Metabolic labeling with [¹⁴C]proline was carried out using serumless proline free RPMI (20). For immunoprecipitation, protease inhibitors and Tris-HCl buffer (pH 7.5, 20 mM final concentration) were added to the medium. The cell layer was rinsed, and cells lysed in 1% sodium deoxycholate in 20 mM Tris-HCl, 140 mM NaCl, pH 7.5 containing protease inhibitors. Medium and cell lysates were centrifuged at 18,000 *g* for 30 min at 25°C and 250 μ l of supernatant immunoprecipitated by adding equal volumes of conditioned hybridoma medium or ascites (1:100 diluted). After incubation for 2 h at 37°C, 125 μ g of rabbit-anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA) was added, and the mixture incubated for 1 h. Then, 50 μ l of a 10% suspension of killed, fixed Cowan strain *S. aureus* (IGSORB; The Enzyme Center, Boston, MA) was added, the mixture incubated for 30 min at 37°C, bound immunoglobulin complexes pelleted at 10,000 *g* for 5 min and rinsed with three cycles of vortexing and sedimentation in 50 mM HEPES-140 mM NaCl containing protease inhibitors, 1% sodium deoxycholate, and 1% Tween-20, pH 7.5. Immunoprecipitation with polyclonal IgG anti-FN was carried out similarly using 25 μ g of purified anti-FN or preimmune IgG. The rinsed IGSORB was suspended in 75 μ l of SDS-sample buffer containing 50 mM dithiothreitol, heated (100°C, 5 min), centrifuged at 10,000 *g* for 1 min, and the supernate removed and analyzed by SDS PAGE and fluorography (20).

Immunofluorescence Staining: Rat lung fibroblasts were fixed with 3.5% paraformaldehyde, pH 7.4, and rinsed in Tris-buffered saline, pH 7.4, containing 1 mg/ml of heat-denatured bovine serum albumin. Cells were permeabilized with 1% Triton X-100 in Tris-buffered saline for 5 min. Subsequent staining steps (e.g., hybridoma-conditioned medium followed by fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG) (Miles Laboratories, Elkhart, IN) were carried out for 30 min at 25°C, followed by three rinses of 5 min each in Tris-buffered saline containing bovine serum albumin. Slides were mounted in Tris-buffered saline containing 1 mg/ml of *p*-phenylene-diamine, pH 8.0, to prevent FITC bleaching. Complete details of our immunofluorescence protocols have been given previously (13).

RESULTS

Immunofluorescence Results

This artifact was discovered by screening hybridoma-conditioned medium containing anti-human plasma FN monoclonals for reaction with rat diaphragm cryostat sections (17). 7 of 35 hybridoma culture supernates cross-reacted with rat diaphragm, but gave a qualitatively different pattern than polyclonal anti-FN. By ELISA, all bound to the elastase-released, carboxy terminal 140,000 mol wt fragment of FN. When we stained IMR-90 human lung fibroblasts with these hybridoma-conditioned media, the results were identical to results obtained with polyclonal anti-FN (cf. reference 17). However, staining of RLF with hybridoma-conditioned media resulted in exclusively intracellular, intense granular perinuclear staining (Fig. 1, *A* and *B*), whereas polyclonal anti-FN resulted in characteristic pericellular matrix and intracellular FN staining (Fig. 1, *C* and *D*). When RLF were not permeabilized before incubation with hybridoma-conditioned media, no staining resulted.

To elucidate the cause of this unusual staining pattern of RLF by hybridoma-conditioned medium, we performed a series of immunostaining experiments. The following results suggested that *serum-derived FN* in the hybridoma-conditioned medium was responsible for the staining: (*a*) Ascites fluid containing the hybridoma antibodies did not stain RLF, but addition of human plasma FN to the ascites resulted in intracellular fluorescence identical to that observed with hybridoma-conditioned medium. (*b*) Mixing ascites with FN purified from hybridoma medium also gave intracellular staining identical to that observed with hybridoma-conditioned medium. (*c*) Ascites mixed with FN-depleted hybridoma medium did not stain RLF; addition of FN puri-

fied from the hybridoma medium to the hybridoma medium-ascites mixture restored staining. (*d*) The exclusively intracellular staining of RLF could be reproduced by staining with a mixture of pure human plasma FN in antigen excess to affinity-purified polyclonal anti-human plasma FN.

The immunostaining results suggested that FN-anti-FN monoclonal IgG complexes were binding to intracellular ligands in fixed, permeabilized RLF. Moreover, based upon immunostaining with ascites or purified antibodies, the monoclonal antibodies to human plasma FN did not cross-react with rat FN. Enzyme digestion experiments carried out on permeabilized and nonpermeabilized cells suggested that collagenous components were the intracellular ligands responsible. Bacterial collagenase devoid of nonspecific proteinase activity (Sigma Type VII, 150 U/ml for 1 h at 37°C) abolished staining with hybridoma-conditioned medium. Neither chondroitinase ABC (Miles Laboratories, 5 U/ml, 37°C, 1 h) nor testicular hyaluronidase (Sigma Chemical Co., St. Louis, MO; 1 mg/ml, 37°C, 1 h) affected staining. Thus, collagenase-sensitive intracellular ligands appeared to be binding FN-anti-FN complexes.

Immunoprecipitation Experiments

Binding of collagenous components to FN-anti-FN complexes was confirmed by a series of immunoprecipitation experiments using metabolically labeled RLF cell lysates and culture medium with polyclonal antibodies, ascites, or hybridoma-conditioned medium. Representative results are depicted in Figs. 2 and 3. Fig. 2, lane *A* is a fluororogram of [¹⁴C]proline-labeled proteins secreted by RLF. FN and two major chains co-migrating with purified pro- α_1 (I) and pro- α_2 (I) (*PC*) are indicated. Lane *B* shows the same sample after bacterial collagenase digestion. Note that FN is not degraded, and two additional polypeptides migrating close to the pro- α_1 (I) chain are revealed. Immunoprecipitation of *A* with polyclonal anti-FN yields only FN (lane *C*), while preimmune IgG precipitates no labeled polypeptides (lane *D*). This experiment indicates that the clostridial collagenase we used is free of FN degrading activity, the polyclonal anti-FN precipitates only FN, and the majority of [¹⁴C]proline-labeled polypeptides secreted by rat lung fibroblasts are collagenous.

Fig. 3 shows the results of a similar experiment using ascites fluid containing monoclonal antibody to human plasma FN. Lane *A* displays the total [¹⁴C]proline-labeled medium polypeptides from RLF. Polyclonal anti-FN immunoprecipitates the small amount of FN present, as well as small amounts of other polypeptides (lane *B*). Neither preimmune IgG (lane *C*) nor ascites containing monoclonal antibody to human FN (lane *D*) precipitated rat FN. Addition of human plasma FN in antigen excess to the ascites precipitated a number of labeled polypeptides (lane *E*), all of which were sensitive to bacterial collagenase (lane *F*). The two major polypeptides were of apparent mol wt 162,000 and 140,000, corresponding to the size of authentic purified procollagen type I chains. By densitometric scanning (across the narrowest dimension of the precipitated polypeptides to avoid errors introduced by differing widths of lanes), we estimated that at least 60% of the total [¹⁴C]proline-labeled polypeptides in medium were precipitated by the monoclonal antibody-FN mixture. The two major labeled polypeptides were present in a ratio of 1.7:1. As shown in lane *F*, pepsin treatment converted the precipitated collagenous peptides to two major polypeptides migrating with apparent mol wt 140,000 and 130,000. These

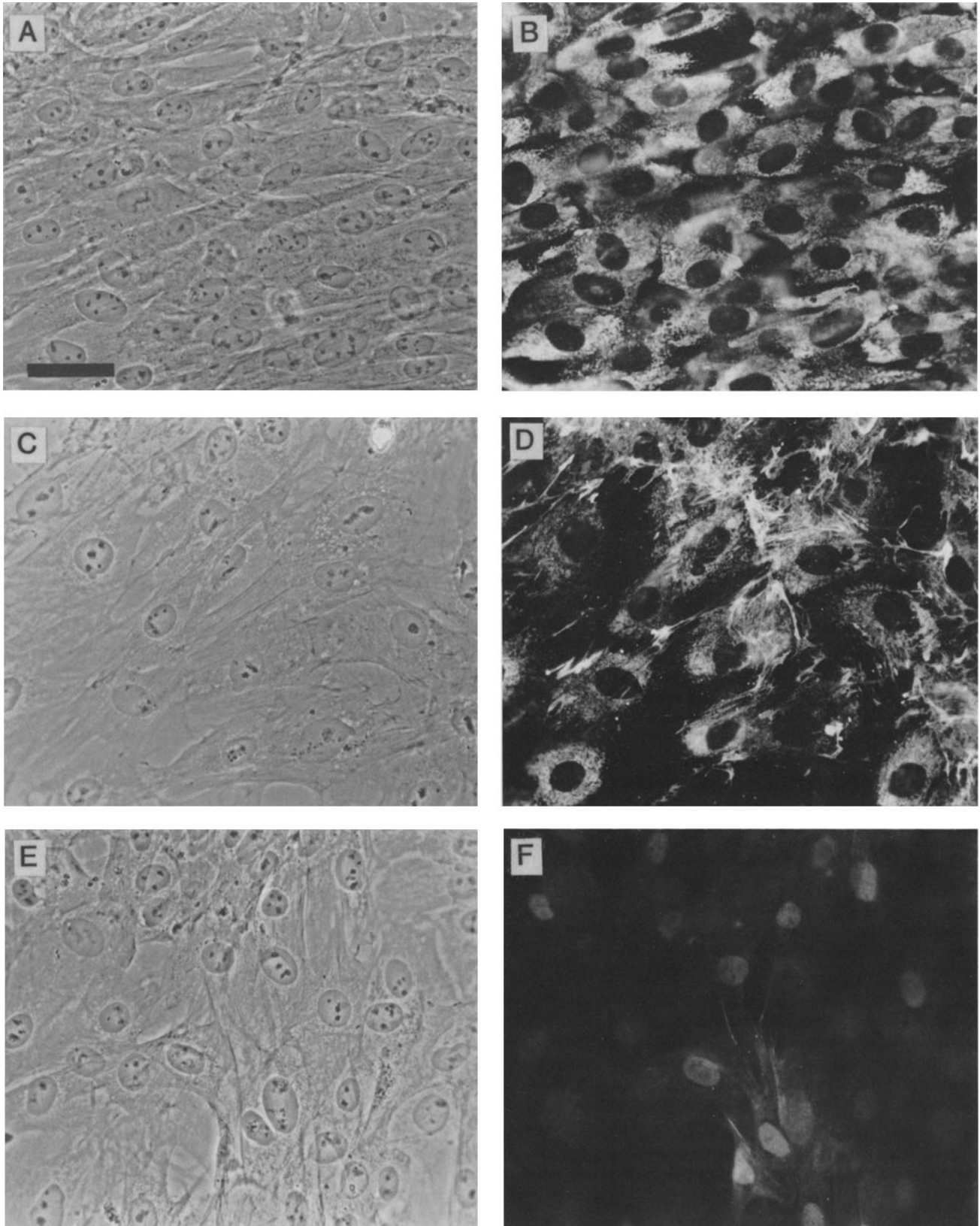


FIGURE 1 Immunofluorescence staining of permeabilized rat lung fibroblasts with hybridoma conditioned medium or polyclonal antifibronectin IgG. The same field is shown by phase contrast and fluorescence. (A and B) Stained with hybridoma conditioned medium. Note the intense intracellular staining and total absence of pericellular matrix staining. (C and D) Stained with affinity purified antibodies to human plasma fibronectin. Despite lower cell density than in A, there is obvious pericellular matrix as well as intracellular staining. (E and F) are Triton-permeabilized cells incubated with purified bacterial collagenase (100 $\mu\text{g}/\text{ml}$, 10 min, 37°C) followed by rinsing and staining as in A and B. Note that only faint nonspecific nuclear fluorescence is seen, indistinguishable from cells incubated with second antibody alone. Bar in A, 50 μm . $\times 300$.

pepsin-resistant chains co-migrated with authentic purified human type I α_1 and α_2 chains. We have displayed results obtained with RLF medium for illustrative purposes, although similar results were obtained with cell lysates. On the basis of similar molecular weights, collagenase sensitivity, pepsin resistance of α -chain sized digestion products of larger precursors, and the fact that fibroblastic cells synthesize procollagens I and III, we tentatively identify these two major polypeptides precipitated by FN-anti-FN complexes as procollagen type I and possibly III α -chains. Obviously further biochemical confirmation is required, although little doubt concerning their collagenous identity remains.

Thus, a mixture of monoclonal antibody to human plasma FN with FN immunoprecipitated collagenous polypeptides, which appeared to be primarily type I procollagen. Identical results were obtained with serum-containing hybridoma culture medium (not shown). To determine whether this pattern of precipitation could be duplicated by other antigen-antibody combinations, we performed additional experiments. First, we reproduced the precipitation experiments substituting polyclonal anti-FN for the monoclonal antibody and adding excess FN. This mixture precipitated the same collagenous polypeptides as the monoclonal antibody-antigen mixture. Second, neither preimmune IgG nor a SP2-derived monoclonal antibody to bovine elastin (generously provided by Dr. Robert Mecham, Washington University Medical School) precipitated collagenase-sensitive polypeptides from rat lung fibroblast medium or cell lysate when compared with preimmune or no primary antibody controls. Third, addition of excess FN to these control antibodies also did not result in significant precipitation.

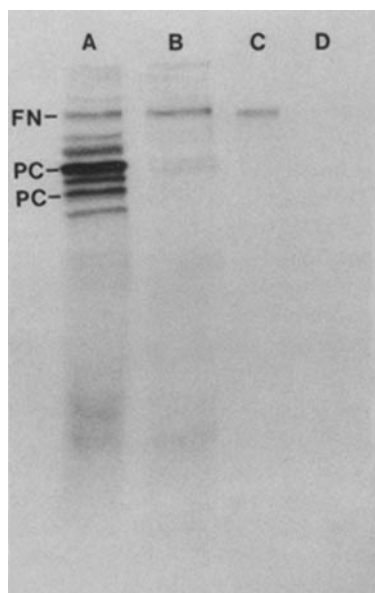


FIGURE 2 Immunoprecipitation of [14 C]proline labeled rat lung fibroblast culture medium with polyclonal antifibronectin. This is a fluorogram of a 7.5% PAGE. Lane A is the total polypeptides, with the fibronectin ("FN") and two major polypeptides co-migrating with purified type I procollagen ("PC") indicated. Lane B shows the result of digesting the sample in A with purified *Clostridial* collagenase. Note that protease-sensitive FN is spared, and two minor bands migrating at approximately the position of pro- $\alpha_1(I)$ are revealed. Lane C is an immunoprecipitate of the sample in lane A with polyclonal anti-FN antibody, while lane D is a corresponding control with preimmune serum.

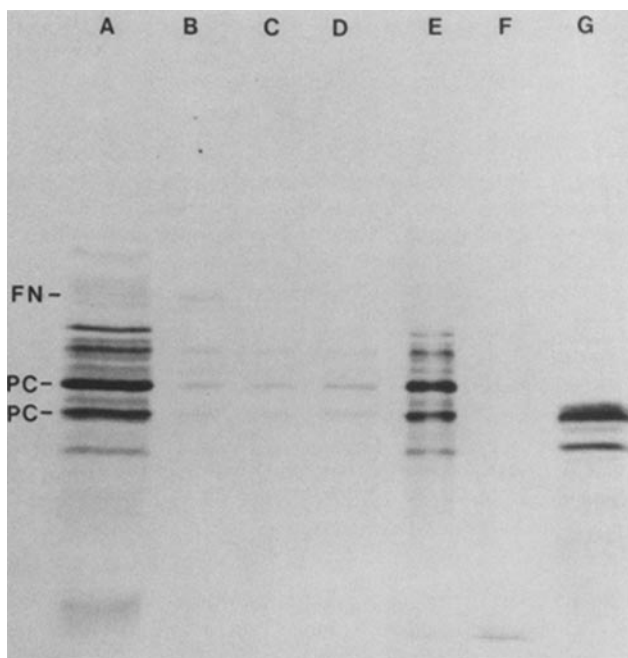


FIGURE 3 Immunoprecipitation of [14 C]proline labeled rat lung fibroblast culture medium with polyclonal antifibronectin. This is a fluorogram of an SDS 5% PAGE, and the fibronectin (FN) and procollagen type I α -chains (PC) are indicated as in Fig. 2. Lane A displays the total secreted labeled polypeptides. Note that FN constitutes a relatively minor component of the secreted, proline-labeled material, most remaining associated with the cell layer (not shown). Lane B is an immunoprecipitate of A with polyclonal anti-FN, lane C is a polyclonal preimmune control, and lane D is an immunoprecipitate with ascites containing anti-human FN monoclonal antibody. There is some nonspecific precipitation of labeled polypeptides corresponding to the major ones in C and D, but no detectable FN. Lane E demonstrates the results of mixing the same concentration of ascites used in lane D with excess human plasma FN. A number of polypeptides are precipitated, with two major components corresponding to the major proline-labeled bands in the medium. Collagenase digestion of the sample in lane E removes all of the labeled polypeptides, while pepsin digestion (lane G) converts them to two major bands corresponding in size to α -chains of type I collagen.

With these immunoprecipitation results in hand, we performed two additional staining experiments to determine which component of the immune complexes (i.e., FN or immunoglobulin) was binding to intracellular ligands. In the first experiment, fixed and permeabilized RLF were incubated with 100 μ g/ml of human plasma FN for 1 h at 37°C, rinsed extensively, and then incubated with ascites followed by FITC-conjugated second antibody (incubation with ascites followed by second antibody did not give intracellular staining). These cells exhibited intense intracellular fluorescence identical to RLF stained with hybridoma-conditioned medium, demonstrating that dimeric human plasma FN bound to intracellular ligands in the fixed and permeabilized RLF. In the second experiment, we attempted to block putative procollagen binding sites on FN by incubating a mixture of ascites and FN with pure heat-denatured type I collagen (2:1 molar ratio of collagen to FN, using 100,000 mol wt for collagen and 220,000 for FN) for 1 h before addition to permeabilized RLF. This blocked subsequent intracellular fluorescence staining with ascites followed by FITC-conjugated second antibody.

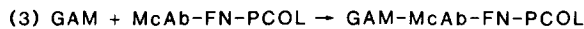


FIGURE 4 Schematic representation of the nonimmunologic interaction of fibronectin immune complexes with intracellular procollagen. Step 1 depicts the binding of monoclonal antibodies (McAb) to fibronectin (FN) to form an immune complex. In our initial experiments, this step occurred during culture of the monoclonals in serum (and thus FN) containing medium. Step 2 depicts the binding of monoclonal antibody-FN complexes to procollagen (PCOL) via the FN to form a monoclonal antibody-FN-procollagen complex. In step 3, the monoclonal antibody is bound by a second antibody, in this case a goat-antimouse (GAM) to yield the final complex. If the second antibody is FITC-conjugated, intense intracellular fluorescence results (see Fig. 1). In principle, this type of interaction could occur with any serum component capable of binding to cellular or secreted antigens.

Based upon the immunoprecipitation and staining experiments, we conclude that "soluble" (the complexes were not centrifuged at sufficient force to use this term as operationally defined) immune complexes of monoclonal or polyclonal antibody with FN, either formed in hybridoma-conditioned medium from FN present in the sera, or created by adding purified FN to ascites fluid or to polyclonal anti-FN IgG, bind to intracellular collagenous components in rat lung fibroblasts via the FN portion of the immune complex. The resulting antibody-collagen complex binds FITC-conjugated second antibody, resulting in intracellular staining. This scheme is depicted in Fig. 4.

DISCUSSION

We encountered a new artifact resulting from the use of hybridoma conditioned medium for immunostaining. By customary immunohistochemical criteria, the intracellular staining of RLF by hybridoma-conditioned medium appeared specific. Appropriately negative controls included no staining: (a) with second antibody alone; (b) with hybridoma medium alone followed by second antibody; or (c) with hybridoma-conditioned medium absorbed with rat plasma FN (this was presumably due to the rat plasma FN blocking intracellular collagenous binding sites for FN but not being bound by the monoclonal antibodies). The only clue to the artifact was the difference in the distribution of tissue staining between polyclonal and monoclonal antibodies.

This artifact is potentially important, as inspection of Fig. 4 reveals that it could occur in any situation in which hybridoma culture medium contains serum antigens that cross-react with a monoclonal antibody and also bind to cellular or tissue ligands. Possible candidates include many serum components such as immunoglobulins and complement factors. Thus, monoclonal antibody staining, especially using hybridoma-conditioned medium in heterologous systems, may be subject to new artifacts of which investigators should be aware (see also reference 8).

Apart from revealing a troublesome immunochemical artifact, our demonstration of intracellular collagenous binding sites for human plasma and cell secreted FN in RLF raises questions about FN-procollagen interaction. Collagen and FN most likely share a spacially common secretory pathway, at least to the level of the Golgi (6, 10, 15), and evidence supports

significant interactions between these matrix components in the extracellular space (c.f. reference 13). Yet, it seems unlikely that intracellular FN-procollagen binding occurs under physiologic conditions, as large multimers hindering transcellular movement could result. Thus, specific regulatory mechanisms may operate to prevent intracellular assembly. Of course, we may simply have circumvented these by fixation and permeabilization. However, alternative possibilities could account for our results. First, FN multimers, resulting in our experiments from antibody binding and physiologically by assembly in the extracellular milieu may have higher avidity for procollagen. This has been previously demonstrated for FN-binding to heparin and hyaluronate (9), and a ternary complex between FN, procollagen(s), and heparan sulfate proteoglycan may be required for tight binding. Posttranslational modifications of FN may function to limit binding to procollagen during synthesis and before secretion. FN is synthesized with asparagine-linked, mannose-rich carbohydrate side chains located on the gelatin binding domain. These high mannose oligosaccharides are replaced with the complex form before secretion (2, 16). As the carbohydrates render FNs collagen binding domain protease resistant and are not required for its secretion by fibroblasts, protease resistance has been speculated to be one of their functions (1). However, replacement of the high mannose with the complex side chain might allow procollagen binding, or FN-FN interaction, which may also be localized to this domain (13). It would be necessary to test binding of the intracellular high mannose form of FN to native procollagen to test this hypothesis.

Previously, it has been observed that plasma fibronectin is rapidly incorporated into the pericellular matrix of cultured fibroblasts (5, 14). However, formalin-fixed RLF pericellular matrix did not decorate with FN-anti-FN immune complexes during a 30-min incubation. This does not contradict previous studies, as we could easily detect human plasma FN in the deoxycholate insoluble pericellular matrix of RLF after coculturing for 24 h. Possibly, live cells are necessary for FN incorporation, or the binding sites are sensitive to formalin fixation. It may appear paradoxical that intracellular but not pericellular matrix collagens bind human FN. However, recent results from our laboratory suggest that collagenous ligands are not responsible for FN binding to fibroblast pericellular matrix. Moreover, it appears that FN is in excess to collagen in matrix deposited early after subcultivation by fibroblasts. For example, IMR-90 human lung fibroblast pericellular matrix contains free FN-derived binding sites for FITC-labeled gelatin that are blocked by Fab' to the FN gelatin-binding domain (reference 13, unpublished observations).

We hope that FN-antibody complexes of defined composition may be useful in studying the molecular basis of pericellular matrix organization. Previously, we have not been able to identify FN-procollagen interaction of reasonable avidity using a variety of measures, including solid phase derivatives such as FN-Sepharose and isolated gelatin-binding domains of FN coupled to Sepharose. As Engvall and co-workers (3) could readily demonstrate native type I collagen-FN interaction using this method, procollagen-FN interaction may be weaker. However, the demonstration of procollagen binding to FN-anti-FN complexes together with our previous study demonstrating inhibition of collagen matrix formation with collagen binding domain specific antibodies suggests that FN-procollagen interaction is physiologically relevant. Nota-

bly, similar immune complexes have been used in the purification of a cellular transferrin receptor, suggesting that this technology may be generally useful for the study of macromolecular interactions (18).

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