Fibronectin Deposition in Delayed-type Hypersensitivity

Reactions of Normals and a Patient with Afibrinogenemia

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Abstract. During development of delayed hypersensitivity (DH) skin reactions, fibronectin accumulates in two distinct sites: (*a*) the dermal interstitium in a pattern similar to fibrin and with a time course similar to that of fibrin deposition and mononuclear cell infiltration, and (*b*) blood vessel walls in a pattern suggestive of basement membrane staining and with a time course similar to that of endothelial cell proliferation. In vitro fibronectin can bind to monocytes or endothelial cells and simultaneously bind to fibrin or collagen matrices; by such interaction in vivo it may affect cell migration or proliferation. Thus, fibronectin deposition in DH reactions may facilitate cell-matrix interactions; however, the possibility exists that extravascular fibronectin accumulation may be only secondary to interstitial fibrin clot formation, and that blood vessel-associated fibronectin may be only a function of adsorption onto basement membrane (type IV) collagen. To address these possibilities, we investigated the association of fibronectin with fibrin, type IV collagen, and mononuclear cell infiltrates in DH reactions. Skin sites of DH reactions in normal volunteers were biopsied at 24, 48, and 72 h after intradermal challenge and examined by immunofluorescence technique. At all time points most of the interstitial fibronectin coincided with fibrin; however, some interstitial fibronectin was coincident with

mononuclear cells positive for HLA-DR or monocyte-specific antigen. The coincidence of fibronectin with mononuclear cells was more apparent in a 48-h DH reaction from a patient with congenital afibrinogenemia. Vessel wall fibronectin was increased by 48 h after challenge and appeared as a fine linear band on the luminal side of a much thicker band of type IV collagen. Thus, the coincidence of extravascular fibronectin with mononuclear cells, its appearance without fibrin in the site from a patient with afibrinogenemia, and incomplete correspondence of vessel wall fibronectin with type IV collagen suggest that fibronectin localization in DH reactions involves endothelial cell and mononuclear cell binding as well as adsorption to fibrin and/or type IV collagen.

Introduction

Fibronectin, a 440-kD glycoprotein, can simultaneously bind to certain cells, including endothelial cells and monocytes, and to biologic substrata, such as collagen and fibrin (1, 2), in vitro and it is associated with these cells as they migrate and/or proliferate into sites of inflammation and wound healing in vivo (3, 4). Using a combination of immunofluorescent and radioisotopic techniques, we have previously demonstrated (4) that plasma-derived fibronectin deposits in the dermal interstitium of 1- and 2-d-old delayed hypersensitivity (DH)[1] reactions in a pattern similar to that of fibrin (5) and a time course similar to fibrin clot deposition and monocyte infiltration (6). By 3 d, a marked reduction in extravascular fibronectin and fibrin occurs, and fibronectin increases in dermal blood vessel walls in parallel with maximal endothelial cell proliferation. In subsequent studies we have demonstrated that microvascular

1. *Abbreviations used in this paper:* DH, delayed-type hypersensitivity.

fibronectin is produced in situ by blood vessels in response to injury (7). These findings suggest that fibronectin may play a role in cell recruitment to sites of inflammation through the modulation of cell migration and/or proliferation.

The skin sites of DH reactions in normal volunteers were studied with fluorescein-labeled antibodies specific for fibronectin, fibrinogen, HLA-DR, monocyte-specific antigen, and type IV collagen to determine the relationship of fibronectin deposits to fibrin, mononuclear cells, and basement membrane collagen. In normal subjects, DH reactions contain abundant deposits of extravascular fibrin, which are lacking in patients with afibrinogenemia (8); therefore, we also studied a subject with congenital afibrinogenemia. We reasoned that if interstitial fibronectin accumulation occurred in the absence of fibrin deposition and if fibronectin increased within the microvasculature in a pattern of nonidentity with type IV collagen, then fibronectin accumulation in these sites could not be entirely attributable to a simple adsorption to fibrin or type IV collagen but must be due to other factors such as inflammatory cell infiltrates and endothelial cells. This outcome would support the hypothesis that fibronectin plays a specific role in cellular proliferation and recruitment to sites of inflammation.

Methods

Subjects and tissue protocol. Three fully informed, consenting normal volunteers (RAFC, CRH, and RBC) and one subject (J.G.) with congenital afibrinogenemia who has been the subject of a previous report (8) were used in this study. In two normal volunteers (RAFC and CRH) 4-mm skin punch biopsies were excised at 24, 48, and 72 h from two sets of positive DH reaction sites challenged intradermally with monilia or mumps antigen and at 48 h from negative DH reaction sites challenged with purified protein derivative. Skin punch biopsies were also excised at 48 h after intradermal challenge with normal saline, 10 μg histamine, and monilia in two normal volunteers (RAFC and RBC) and mumps antigen in the patient with afibrinogenemia. Positive DH reactions in controls were 15–20 mm of induration at all time periods examined, with no evidence of necrosis. No induration but 21 mm of erythema occurred at the mumps DH reaction site in the afibrinogenemia subject (8). Each specimen was bisected and one-half was snap frozen in liquid N_2 for immunofluorescence studies. Fluorescent antibody technique was performed as previously described (4).

Antibody production, purification, conjugation, and specificity. Human fibronectin was purified by a modification (4) of the gelatin affinity column technique (9). The purity of fibronectin was established by sodium dodecyl sulfate-polyacrylamide slab gels and immunoelectrophoresis against anti-human serum (4). Antibodies to fibronectin
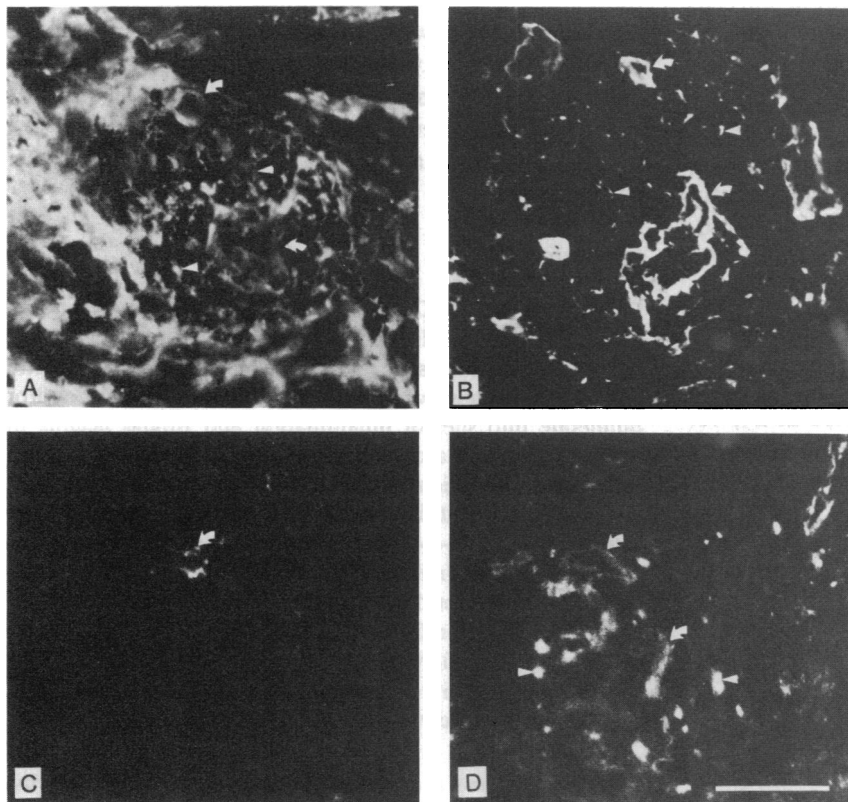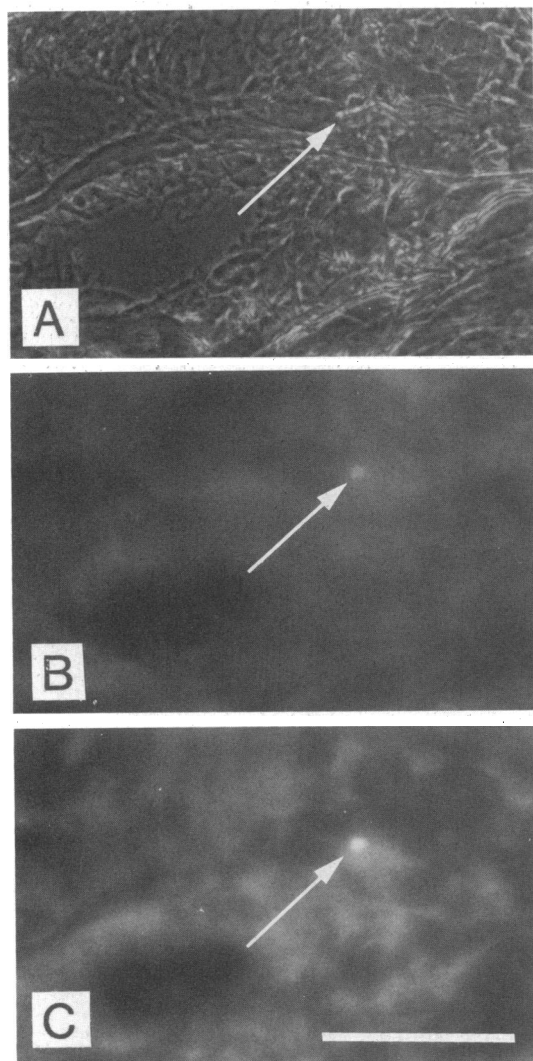

Figure 1. Immunofluorescence study of frozen skin specimens from 48-h DH reaction sites on a normal volunteer (*A* and *B*) and a patient with afibrinogenemia (*C* and *D*). (*A*) Area around mid-reticular blood vessels was stained with fluorescein-conjugated F(ab')$_2$ anti-human fibrinogen. Vessels are weakly fluorescent (arrows). Fine fibrillar staining is seen immediately around the vessels (arrowheads) in a location identical to the perivascular infiltrate identified under phase-contrast microscopy (not shown). Intensely stained broad fibrils of fibrin are seen in the more remote dermis. (*B*) Adjacent tissue section stained with a 1:4 dilution of fluorescein-conjugated anti-human fibronectin. Vessels are brightly stained for fibronectin (arrows). Fine short fibrillar staining is seen immediately around the vessels (arrowheads) in a location identical to the perivascular infiltrate and fibrin (*A*). Intensely stained broad fibrils of fibronectin are seen in a pattern and location similar to fibrin (*A*). (*C*) Area around upper reticular blood vessels stained with fluorescein-conjugated F(ab')$_2$ anti-human fibrinogen. Staining is confined to a small portion of one blood vessel wall possibly secondary to platelets (arrow). (*D*) Adjacent tissue section stained with 1:8 dilution of fluorescein-conjugated anti-human fibronectin. Vessels are moderately stained for fibronectin (arrows). Globular staining is seen around the vessels (arrowheads) in a location that corresponds to the perivascular infiltrate under phase-contrast microscopy. Bar, 60 μm.
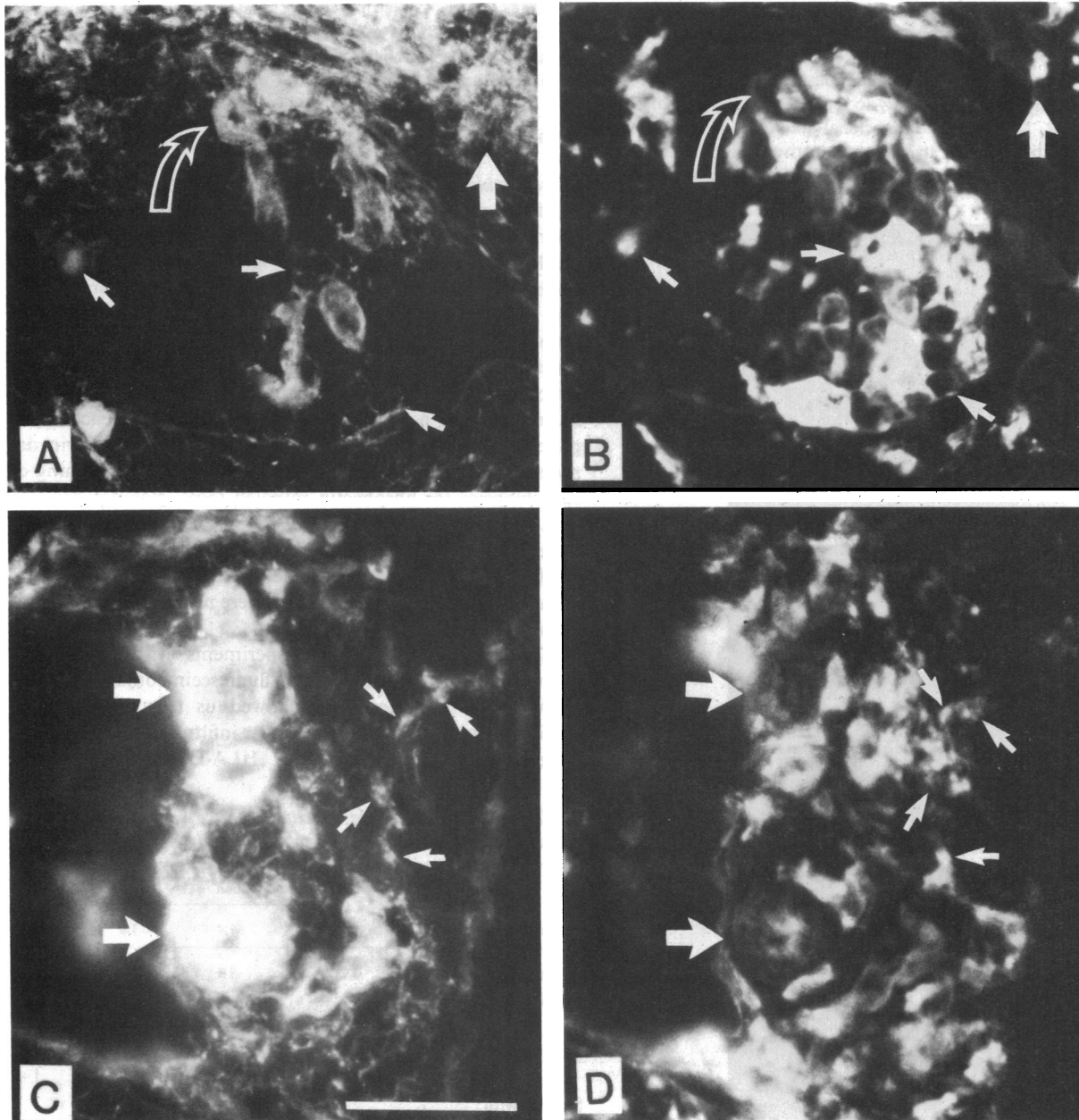
Figure 2. Double-label immunofluorescence study of frozen skin specimens from a normal volunteer at 24 h (*A* and *B*) and 72 h (*C* and *D*) using rhodamine anti-human fibronectin (*A* and *C*) and fluorescein-conjugated monoclonal anti-human HLA-DR (*B* and *D*) (*A*) Area around mid-reticular blood vessels viewed with rhodamine filters shows weak fibronectin staining of blood vessels (open arrow), bright reticular staining of interstitium away from vessels (large arrow), and patchy weak globular and reticular staining of inflammatory infiltrate around vessels (small arrows). (*B*) Same specimen viewed with fluorescein filters shows intense staining of inflammatory infiltrate around vessels. Small arrows demonstrate cells stained by both fibronectin (see *A*) and HLA-DR. Large arrow indicates cell in interstitium away from vessels that stains for both fibronectin (see *A*) and HLA-DR. Open arrow shows blood vessel whose endothelial cells stain only for HLA-DR while the vessel wall stains only for fibronectin (see *A*). (*C*) Area around mid-reticular blood vessels viewed with rhodamine filters shows intense staining with antifibronectin (large arrows). Small arrows point to cells of inflammatory infiltrate that stain for both fibronectin and HLA-DR (see *D*). (*D*) Same specimen viewed with fluorescein filters show staining of endothelial cells in some (lower large arrow) but not all (upper large arrow) blood vessels. Small arrows denote cells that stain for both fibronectin (see *C*) and HLA-DR. Bar, 80 μm.

were produced, isolated, and tested for specificity as previously described (4). Rabbit F(ab')₂ anti-human fibrinogen (Cappel Laboratories, Cochranville, PA) was free of anti-fibronectin contaminants as determined by immunoelectrophoresis and lack of staining of W138 fibroblasts. A monospecific affinity-purified antibody to type IV collagen was kindly provided by G. R. Martin (10). Monoclonal antibodies to the HLA-DR framework antigen, which stains B cells, activated T cells, monocytes, and dendritic cells were obtained from Becton-Dickinson & Co.

Figure 3. Double-label immunofluorescence study of frozen skin specimen from a normal volunteer at 72 h stained with rhodamine-conjugated F(ab')₂ anti-human fibronectin (*C*) and mouse monoclonal IgG anti-Mono 0.2 followed by fluorescein-conjugated affinity purified goat anti-mouse IgG (*B*). (*A*) Phase contrast of mid-reticular interstitium away from vessels. Arrow demonstrates mononuclear cell adjacent to collagen bundle. (*B*) Same field viewed with fluorescein filters shows that the cell stains for anti-Mono 0.2 (arrow). (*C*) Same field viewed with rhodamine filters shows that the cell stains for anti-fibronectin. Bar, 80 μm.

(Mountain View, CA), while monoclonal antibodies to a monocyte-specific antigen (Mono 0.2) was purchased from BRL Laboratories (Bethesda, MD). Antibodies were conjugated with fluorescein or rhodamine by the dialysis method (4).

Results

Extravascular fibrinogen and fibronectin. As previously reported (5), fibrin and/or fibrinogen were deposited extensively in the interstitial space of the mid-reticular dermis 24–72 h after skin challenge in sensitized subjects (Fig. 1 *A*) in parallel with the observed induration of skin test site. When the same tissue was stained with fluorescein-conjugated F(ab')₂ antibodies to human fibronectin, the extravascular regions of the mid-reticular dermis showed a similar reticular pattern of staining, which was somewhat less extensive and less intense (Fig. 1 *B*). In contrast, the nonindurated, erythematous DH reactions in patients with congenital afibrinogenemia had little fibrin deposition (10), a finding confirmed here (Fig. 1 *C*). Staining of such biopsies for fibronectin revealed focal, globular deposits in a perivascular array (Fig. 1 *D*) instead of the reticular pattern found in normal DH reactions (4, Fig. 1 *B*). Negative purified protein derivative challenge sites as well as normal saline and histamine injection sites were negative for interstitial fibrin and fibronectin

Double-antibody binding experiments with rhodamine-conjugated anti-fibronectin and fluorescein-conjugated anti-HLA-DR or anti-Mono 0.2 allowed us to examine tissue specimens for fibronectin and cellular infiltrate simultaneously. At each time interval observed, HLA-DR and fibronectin

*Table I. Superficial Venular Plexus Staining with Fluorescein-conjugated Anti-Human Fibronectin Antibody*

Human skin site	Antibody dilution			
	1:2	1:4	1:8	1:16
Normal saline*	2+	1+	—	—
Intradermal histamine (1 h)*	2+	—	—	—
DH reaction to mumps (48 h)*	4+	3+	2+	1+
Subject with afibrinogenemia DH reaction to monilia (48 h)	ND	ND	2+	ND

Intensity of fluorescent staining of the superficial venular plexus with various dilutions of fluorescein-conjugated anti-human fibronectin: 1+, definite but weak staining; 2+, moderate staining; 3+ bright staining; 4+, intense staining. ND, not done.
* Composite data from the skin sites of two normal volunteers (RAFC and RBC) into which 0.1 ml of normal saline, 10 μg histamine and mumps antigen had been injected intradermally at 48, 1, and 48 h, respectively, before 4-mm punch biopsies.

often occurred coincidentally in the perivascular area of DH reaction sites (Fig. 2 A–D) and rarely in areas away from blood vessels (not shown). Many fewer cells of the perivascular infiltrate (<10%) stained with antibodies to Mono 0.2 antigen but approximately half of these also stained for fibronectin (Fig. 3 A–C).

Vessel-associated fibronectin. As we observed earlier in guinea pig DH reactions (4), the microvasculature in DH reaction sites of normals and the subject with afibrinogenemia showed increased fibronectin in blood vessel walls as determined by the intensity of staining observed at a given antibody titer and by positive staining at the higher dilutions of antifibronectin antibody (Table I). When these vessels were examined by a double immunofluorescence stain technique by using rhodamine conjugated anti-human fibronectin and fluorescein conjugated anti-type IV collagen, a difference in disposition of fibronectin and type IV collagen was noted in the vessel wall
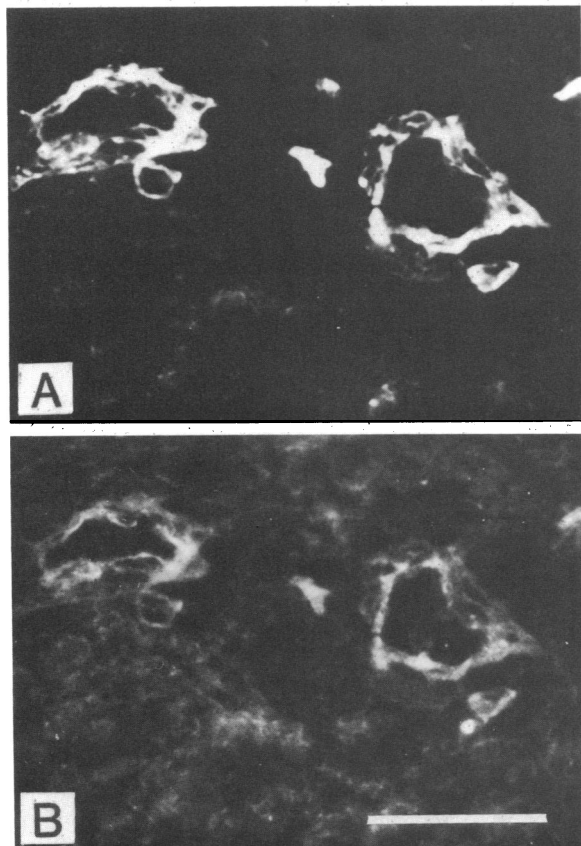
Figure 4. Immunofluorescence study of mid-reticular blood vessels in frozen skin specimens from a 48-h DH reaction site in a normal volunteer. (*A*) Specimen stained with rhodamine-conjugated anti-type IV collagen shows a wide band of fluorescence in the vessel walls. (*B*) Same specimen stained also with fluorescein-conjugated anti-human fibronectin shows a thin rim of fluorescence around the luminal side of the vessel walls. Bar, 80 μm.

(Fig. 4 *A* and *B*). Fibronectin appeared as a fine linear band on the luminal side of a much thicker band of type IV collagen. The dilution endpoint of anti-fibronectin antibody was used to stain the specimen shown in Fig. 4 to facilitate viewing the fine linear staining of the luminal side of the vessel wall. This finding is obscured when higher antibody concentrations are used (see Figs. 1 *B* and 2 *C*).

Discussion

The presence of increased fibronectin in a DH reaction of a subject with congenital afibrinogenemia suggests that both intra- and extra-vascular fibronectin accumulation are at least partially independent of fibrin deposition. The extravascular fibronectin, however, occurred in focal globular deposits instead of the reticular-nodular pattern found in normals. Interestingly, these globular deposits seemed to coincide with the presence of large mononuclear cells when the same sections were examined under phase microscopy. When DH specimens from normals were double labeled with antibodies to fibronectin and either HLA-DR or Mono 0.2 antigen, the coincidental staining of some cells with fibronectin and HLA-DR or Mono 0.2 suggests that fibronectin is associated with monocytes and perhaps dendritic cells (11). Since fibronectin enhances the adherence of monocytes to biological substrata (2) and its fragments are chemoattractants for monocytes (12), fibronectin may be critical for the recruitment of monocytes to areas of inflammation. The lower percentage of monocytes (<10%) observed by us in these reactions compared with 12–30% seen by others (13) is probably secondary to the differences in immunofluorescence techniques.

Double immunofluorescence staining of normal DH reaction sites demonstrated that fibronectin and type IV collagen occupied distinctly different sites in blood vessel walls. Fibronectin always appeared as a thin linear band on the luminal side of a much thicker band of type IV collagen. Therefore, since endothelial cells produce fibronectin in response to injury (7), adhere to fibronectin in tissue culture (1), and migrate in response to fibronectin in vitro (14), it is tempting to speculate that fibronectin provides a scaffolding for proliferating and migrating endothelial cells in sites of inflammation and wound healing.

Acknowledgments

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