

MONOCYTE RECEPTORS FOR FIBRONECTIN
CHARACTERIZED BY A MONOCLONAL ANTIBODY THAT
INTERFERES WITH RECEPTOR ACTIVITY

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Human peripheral blood monocytes express plasma membrane receptors for fibronectin that is bound to substrates such as denatured collagen, gelatin, and fibrin (1). After attachment to surfaces containing fibronectin, monocytes express physiological characteristics usually attributed to inflammatory macrophages (2). Immune phagocytic activity is enhanced, as indicated by increased uptake of particles opsonized by IgG and C3 (1) and the engagement of C3 receptors in phagocytosis (3). In addition, secretion of neutral proteases such as plasminogen activator and elastase after a phagocytic stimulus is enhanced (4). Fibronectin receptors thus appear to be involved in the differentiation of blood monocytes into more active, inflammatory cells. In vivo, at a site of injury, these cells would be more effective in the clearance of debris and microorganisms in preparation for tissue reconstruction.

We have previously described the basic features of fibronectin receptors of human monocytes (1). These receptors are present on most, if not all, human peripheral blood monocytes (5) and on peritoneal macrophages (6, 7), alveolar macrophages (8), and macrophage cell lines (9). Receptors can be detected by their ability to mediate the attachment of monocytes to fibronectin-containing surfaces or by the attachment of fibronectin-coated particles to monocytes (1). The interaction of receptor and ligand requires Mg^{++} and is reversed by chelation of this divalent cation with EDTA. Both monomeric (190–235 kD) and dimeric (450 kD) forms of plasma fibronectin promote monocyte attachment to gelatin surfaces. Soluble fibronectin, however, interacts poorly with the receptor. We have also found that trypsinization abolishes the ability of monocytes to bind to fibronectin-gelatin surfaces, suggesting that a protein-containing structure on the cell surface is involved in receptor activity.

In the present paper we describe a molecule on the surface of human peripheral blood monocytes that appears to be a plasma membrane receptor for fibronectin. This protein has been identified by using a monoclonal antibody, A6F10, that prevents the interaction between monocytes and substrate-bound fibronectin. Thus, at least functionally, the antibody appears to recognize the plasma membrane receptor for fibronectin. We define the properties of this monoclonal

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antibody and report the biochemical characteristics, distribution, and cellular specificity of the surface antigen it recognizes.

Materials and Methods

Reagents. The following materials were obtained from commercial sources: polyethylene glycol (J. T. Baker Chemical Co., Phillipsburg, NJ); EDTA and sodium azide (Fisher Scientific Co., Pittsburgh, PA); protein A from *Staphylococcus aureus*, gelatin, bovine serum albumin (BSA),¹ aprotinin, phenylmethylsulfonyl fluoride (PMSF), polyvinylpyrrolidone, and Ficoll type 70 (Sigma Chemical Co., St. Louis, MO); Hypaque-Ficoll (Pharmacia, Inc., Piscataway, NJ); papain (Worthington Biochemical Corp., Freehold, NJ); Enzymobeads (Bio-Rad Laboratories, Richmond, CA); fetal calf serum (FCS) (Irvine Scientific, Santa Ana, CA); Dulbecco's modified Eagle's medium (DME) (Gibco Laboratories, Grand Island, NY); mouse IgG (Miles Laboratories Inc., Naperville, IL); and F(ab')₂ fragments of goat antiserum to mouse IgG and its fluorescein isothiocyanate (FITC)-conjugated form (Tago, Inc., Burlingame, CA). Fibronectin was prepared from human plasma either by heparin precipitation and ion exchange chromatography, as previously described (10, 11), or by affinity chromatography on gelatin-Sepharose columns (12). The latter material was obtained from the New York Blood Center Blood Derivatives Program. Both preparations were highly purified, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of Human Peripheral Blood Monocytes. Mononuclear cells were obtained from peripheral blood of volunteer donors. The blood was collected in 10 mM EDTA, and mononuclear cells were fractionated by density gradient centrifugation in Hypaque-Ficoll (13). The cells were resuspended in DME and washed by centrifugation. Platelets were removed by low speed centrifugation.

Monocytes were purified by adhesion to fibronectin-gelatin-coated plates, as described below (monocyte-binding assay); nonadherent cells were removed by washes with DME, and the adherent monocytes released by incubation with 5 mM EDTA. This cell suspension was composed almost exclusively (99%) of monocytes, as assessed by nonspecific esterase staining (14) and by phagocytic assays using sheep erythrocytes coated with rabbit IgG and mouse complement (15).

Preparation of Neutrophils. Neutrophils were prepared from human venous blood anticoagulated with EDTA by density gradient centrifugation in Hypaque-Ficoll, followed by dextran sedimentation (16). Residual erythrocytes were eliminated by hypotonic lysis in 0.2% sodium chloride.

Preparation of Buffy Coat Leukocytes. Whole blood was diluted 1:1 with phosphate-buffered saline (PBS) and centrifuged at 1,800 rpm for 30 min. The leukocyte layer was collected and subjected to dextran sedimentation and hypotonic lysis as above.

Monocyte-binding Assay. The binding of monocytes to gelatin-fibronectin-coated plastic surfaces was performed as previously described (1). Briefly, 10–80 µg of purified fibronectin was added to gelatin-coated wells of 24-well tissue culture plates for 1 h at room temperature. After the wells were washed three times with PBS with ions, 10⁶ mononuclear cells in 0.5 ml DME with 1% BSA were added to each well and the plates incubated for 30 min at room temperature in 5% CO₂. The wells were washed three times with HBSS (Hanks' balanced salt solution with calcium and magnesium ions) to remove nonadherent cells, and the monocytes were detached by addition of 1 ml 10 mM EDTA in HBSS without ions. After 10 min of gentle agitation at 4°C, released cells were counted in a Coulter Counter (model Z; Coulter Electronics, Inc., Hialeah, FL). For inhibition assays, cells were preincubated for 30 min at 4°C with the appropriate antibodies, washed once by centrifugation, and added to the wells coated with gelatin and fibronectin.

¹ *Abbreviations used in this paper:* BHK, baby hamster kidney; BSA, bovine serum albumin; DME, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HAT, hypoxanthine, aminopterin, thymidine; HBSS, Hanks' balanced salt solution with Ca⁺⁺ and Mg⁺⁺; HPLC, high performance liquid chromatography; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

Latex Bead Binding Assay. Gelatin-coated latex beads were prepared as previously reported (1). Briefly, washed beads were suspended in PBS containing heat-liquefied gelatin (10 mg/ml) and incubated at room temperature for 2 h. The beads were washed four times and resuspended in DME at 3×10^7 beads/ml. Mononuclear cells (2×10^4) in 3% human serum were allowed to attach to microtiter wells at 37°C for 1 h in an atmosphere of 5% CO₂. Nonadherent cells were removed by washing with DME. Supernatant (10 μ l) from hybridoma cultures was incubated with the cells for 30 min at room temperature. The cells were then washed with DME and incubated with gelatin-fibronectin-coated latex beads at 37°C for 1 h. The cells were washed with DME and fixed with glutaraldehyde before counting of rosettes, defined as more than two beads per monocyte.

Binding of IgG and Complement-coated Sheep Erythrocytes. This assay was performed as previously described (15). IgG-coated sheep erythrocytes or IgM and complement-coated erythrocytes were prepared by standard procedures (15) using mouse serum as a source of complement. Indicator erythrocytes were prepared on the day of use and suspended at 1×10^8 cells/ml in DME.

Preparation of Monoclonal Antibodies. BALB/c mice, 4–6 wk old, were immunized according to the following schedule: 7.5×10^6 monocytes were injected intraperitoneally, followed by injections of 2.2×10^7 mononuclear cells at 3-wk intervals. 4 d after the final injection, the spleen from one animal was removed and teased. Washed spleen cells were fused with washed P3U1 mouse myeloma cells at a 5:1 spleen/myeloma cell ratio using 30% polyethylene glycol (PEG 1000) dissolved in DME (17). The cells were distributed into 96-well plates at 2×10^5 per well in DME with 10% heat-inactivated FCS and hypoxanthine, aminopterin, thymidine (HAT) medium (18), and fed every 5 or 6 d.

Monoclonal antibodies were screened as follows: 96-well plates were coated with gelatin and washed. Fibronectin (0.1 ml of a 50 μ g/ml solution in PBS with calcium and magnesium ions) was added to each well and incubated for 1 h at room temperature. The plates were washed three times with PBS with ions. Human peripheral blood mononuclear cells (2×10^5) were preincubated with 100 μ l of hybridoma supernatant and 50 μ l of DME at room temperature for 10 min in 5% CO₂. A 100 μ l aliquot of this cell suspension was then transferred to the gelatin-fibronectin-coated plate. The plates were incubated for 30 min at room temperature in an atmosphere of 5% CO₂. Nonadherent cells were removed by washing, and adherent cells were fixed with 1.25% glutaraldehyde in PBS and examined by phase microscopy. Supernatants that inhibited the adherence of monocytes to the gelatin-fibronectin surface were considered positive.

Clones were screened for IgG production by immunofluorescence of antibody bound to monocytes, using rhodamine-conjugated anti-mouse IgG. Hybridomas were cloned by limiting dilution and by the soft agar technique according to a modification of the procedure of Coffino et al. (19); mouse peritoneal macrophages containing 2.5×10^6 cells were prepared 48 h in advance of cloning and used as feeder layers for the cloned hybridomas.

Ascitic fluid containing monoclonal antibodies was prepared by injection of $2-5 \times 10^6$ hybridoma cells into BALB/c mice primed 1 wk in advance with 0.5 ml of 2,6,10,16-tetramethyl-pentadecane. The ascitic fluid was tapped every 2 d, centrifuged at 2,000 rpm for 10 min, and frozen at -20°C in the presence of aprotinin and sodium azide.

A6F10 was purified from ascitic fluid by precipitation with ammonium sulphate (50% final concentration) followed by dialysis against 0.02 M Tris buffer, pH 8. This material was then chromatographed on DE-52 and the Ig fraction was eluted using a linear gradient of 0–200 mM sodium chloride in 0.02 M Tris, pH 8. The antibody was also purified by high pressure liquid chromatography (HPLC), using a TSK 3000 column (LKB Instruments, Inc.), Gaithersburg, MD). Fab fragments of A6F10 were prepared by digestion of the purified IgG with a 1:250 (wt/wt) ratio of papain in the presence of cysteine for 22 h at 37°C (20). Fc fragments and uncleaved molecules were removed by ion exchange chromatography on DE-52 in 0.02 M Tris-Cl, pH 8.0, using a 0–300 mM gradient of sodium chloride.

Determination of mouse Ig subclass was made by radial immunodiffusion using antibodies to mouse IgG1, IgG2a, and IgG2b. Quantitation of IgG1 was obtained using

purified standards. Ig concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) (21) using alkaline phosphatase-conjugated rabbit antibodies to mouse IgG.

Indirect Immunofluorescence. Mononuclear cells (4×10^5) in 100 μ l of DME containing 10% human serum were allowed to attach to round (13 mm) glass coverslips for 30 min at 37°C in 5% CO₂. Nonadherent cells were removed by washing with DME. 100 μ l of antibody dilutions were added to the coverslips and incubated in ice for 30 min. Coverslips were then washed three times in ice-cold DME once in PBS containing 2% FCS and 0.01% sodium azide, and once in PBS. FITC-conjugated, affinity-purified F(ab')₂ fragments of goat antibodies to mouse IgG, which had been spun for 30 min at 15,000 g, were added to each coverslip for 30 min. Coverslips were washed twice with PBS, fixed with ice-cold 4% paraformaldehyde in PBS at pH 7 for 10 min, and then washed twice more with PBS. Cells were viewed with a 100 \times oil immersion lens on a Nikon epifluorescence microscope.

Flow Cytometry. Human leukocytes (2×10^6) were incubated for 45 min at 4°C with shaking in 400 μ l of DME containing 20 μ g/ml A6F10 or 50-fold diluted ascitic fluid. The cells were washed twice with PBS containing 2% FCS and 0.01% sodium azide and then resuspended in the above buffer containing a 1:5 dilution of F(ab')₂ fragments of goat antiserum to mouse IgG that had been absorbed with human red blood cells. After a 45 min incubation at 4°C with shaking, the cells were washed twice and resuspended in 1 ml of the above buffer. Fluorescence was analyzed in a FACS IV flow cytometer (Becton Dickinson Immunocytometry, Mountain View, CA) at a gain of 8, laser power of 300 mW, and photomultiplier tube voltage of 860 V.

SDS-PAGE. Cells were lysed in 0.5% Nonidet-40 (NP-40) in the presence of PMSF and aprotinin. Nuclei were removed by centrifugation. Samples were electrophoresed on discontinuous pH, 5–15% polyacrylamide gradient slab gels (22).

Electrophoretic Transfer of Proteins from SDS-Polyacrylamide Gels to Nitrocellulose Sheets. Western blots were prepared according to previously described procedures (23, 24). Electrophoretic transfer was performed at 300 mA for 4 h at 4°C. Nitrocellulose sheets were soaked overnight in 10 mM Tris, pH 7.4, 2 mM EDTA, 2% BSA, 2% Ficoll type 70, and 2% polyvinylpyrrolidone (40T), and then washed three times for 1 h in PBS. A6F10 ascites fluid diluted 1:20 in washing solution (10 mM Tris, pH 7.4, 0.14 M NaCl, 0.5% BSA, 2 mM EDTA, and 0.25% NP-40) was incubated with the electroblots for 4 h. Electroblots were washed three times for 30 min and once overnight. F(ab')₂ fragments of a goat antiserum to mouse IgG, iodinated using Enzymobeads (Bio-Rad Laboratories), were added to the strips at 10⁵ cpm/cm² and incubated for 3 h. The strips were washed extensively, dried at room temperature, and exposed to x ray film.

Isoelectric Focusing. Isoelectric focusing was performed on 1 mm polyacrylamide gels using ampholines in the pH range 3.5–9.5 (25).

Results

Monoclonal Antibody A6F10. Two separate fusions of spleen cells from mice immunized with human peripheral blood monocytes yielded two clones which produced antibodies that inhibited the binding of monocytes to fibronectin-gelatin surfaces; one of these, A6F10, was stable. A6F10 was recloned twice by limiting dilution and once in soft agar. In the latter procedure, clones were selected both for the inhibition of monocyte binding to fibronectin and for increased IgG production.

Biochemical Characteristics of A6F10. A6F10 was purified by several procedures (see Materials and Methods). The antibody elutes from DEAE cellulose at a conductivity of 4.3 mS. In both immunoelectrophoresis and radial immunodiffusion assays, A6F10 was shown to bear determinants of IgG1 mouse Ig. In

isoelectric focusing, A6F10 showed a pI of 6.6 (Fig. 1). A6F10 does not bind to protein A-Sepharose columns of pH 7.0–8.2.

Inhibition of Monocyte Attachment. All clones and subclones derived from A6F10 were tested for the ability to inhibit binding of monocytes to plates coated with gelatin and fibronectin. The inhibition of binding was dependent on the concentration of IgG (Fig. 2). Maximal inhibition occurred at $\sim 1 \mu\text{g}$ IgG₁/ml. Equivalent concentrations of ascitic fluid from mice producing irrelevant antibodies had no effect.

Fab fragments prepared from purified A6F10 IgG were also effective inhibitors of monocyte attachment. In the experiment depicted in Fig. 3, the gelatin surface was preincubated with various amounts of fibronectin. Complete inhibition of monocyte binding was observed at all concentrations, indicating that the Fab fragments of the antibody were effective even when many fibronectin sites were available for cell attachment. This experiment also indicated that the activity

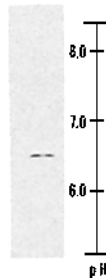


FIGURE 1. Isoelectric focusing of the monoclonal antibody A6F10. A6F10 IgG was analyzed by isoelectric focusing. The pH gradient determined by surface electrode is indicated at right.

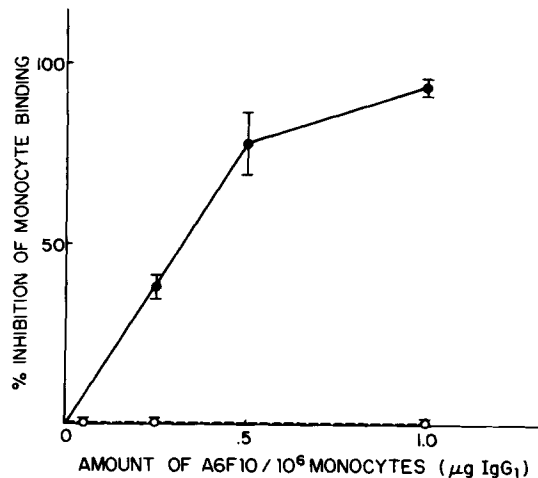


FIGURE 2. The monoclonal antibody A6F10 inhibits monocyte attachment to fibronectin-gelatin surfaces. Human peripheral blood monocytes were incubated with increasing amounts of A6F10 antibody for 30 min at 4°C. Cells were then centrifuged, resuspended in DME, added to fibronectin-gelatin plates, and incubated for 30 min at room temperature. Nonadherent cells were removed by washing, and adherent cells were detached with EDTA and counted. The dotted line represents the activity of a monoclonal antibody of the IgG₁ subclass of undetermined specificity. Vertical bars represent \pm SEM.

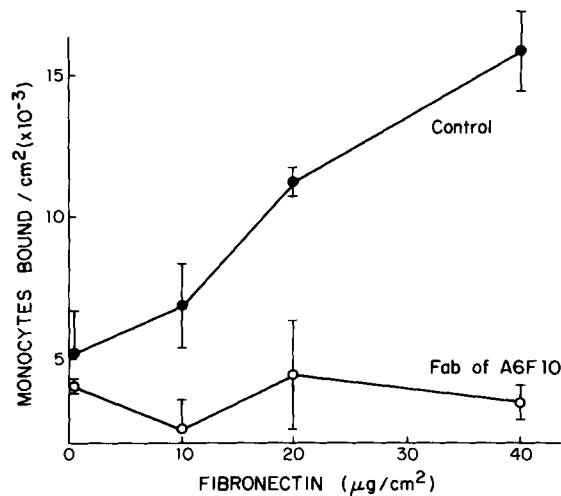


FIGURE 3. Fab fragments of the monoclonal antibody A6F10 inhibit monocyte attachment to gelatin surfaces containing fibronectin. Human peripheral blood monocytes were preincubated with $37 \mu\text{g}$ of Fab of A6F10 as in Fig. 1. The cells were then plated on gelatin surfaces containing varying amounts of fibronectin. The number of adherent cells was determined as in Fig. 1. Vertical bars represent \pm SEM.

of A6F10 is related to its antibody specificity and not simply to interactions mediated by the Fc portion of the molecule. A6F10 also completely inhibited the attachment of gelatin-fibronectin-coated latex beads to monocytes (results not shown).

Other Properties of A6F10. Neither A6F10 nor its Fab fragments had any effect on the attachment of human peripheral blood monocytes to plastic or glass surfaces, on rosette formation between monocytes and sheep erythrocytes coated with IgG and/or C3, or on the phagocytic uptake by monocytes of these opsonized erythrocytes or of $1.1\text{-}\mu\text{m}$ latex beads. These experiments indicated that A6F10 does not react with monocyte receptors for Fc of IgG, with receptors for C3, or with the nonspecific receptors involved in phagocytosis of latex beads. A6F10 was not toxic to monocytes in culture for periods up to 1 h at 37°C . In the presence of rabbit complement, A6F10 was unable to mediate lysis of monocytes, as assessed by trypan blue exclusion.

A6F10 did not react in Ouchterlony assays or in ELISA with human serum proteins, purified fibrinogen, or purified fibronectin. Monoclonal antibodies directed against fibrinogen and fibronectin were used as positive controls in these experiments.

Binding of A6F10 to Blood Cells. Fluorescence microscopy revealed that both A6F10 IgG and its Fab fragments bound only to peripheral blood monocytes. Intracellular staining was never observed. When these experiments were performed at 4°C in the presence of sodium azide, fluorescence appeared as a ring of small patches in the external circumference of the cells (Fig. 4). Staining of lymphocytes, neutrophils, platelets, or erythrocytes was not observed in fluorescence microscopy. A6F10 did not stain human dendritic cells (personal communication, R. Steinman, The Rockefeller University, New York). A6F10 did stain

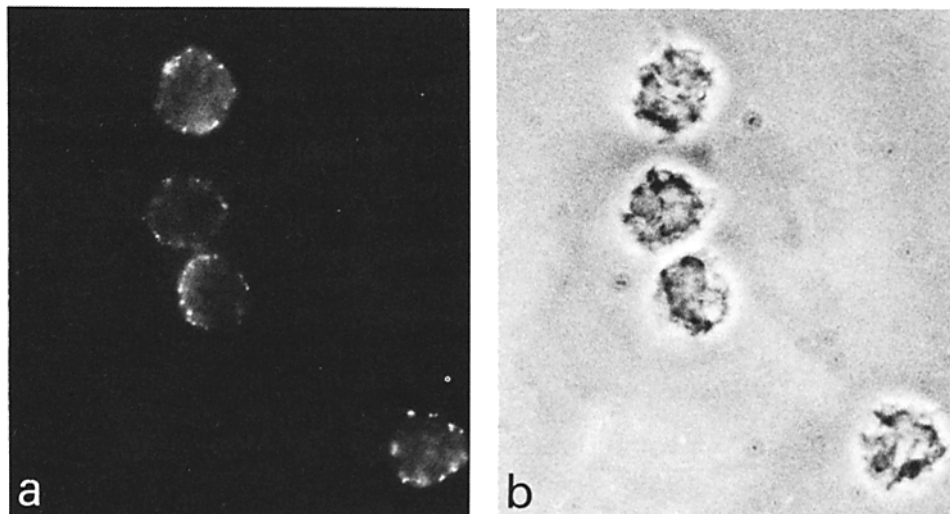


FIGURE 4. Indirect immunofluorescence staining of human peripheral blood monocytes coated with the monoclonal antibody A6F10. Human peripheral blood monocytes purified by Hypaque-Ficoll and adherence to glass coverslips were incubated with A6F10 ascites, followed by FITC-conjugated F(ab')₂ fragments of a goat antiserum to mouse IgG, as described in Materials and Methods. (a) Fluorescence micrograph. (b) Phase contrast micrograph of the same field. $\times 1,000$.

tissue sections of human tissue macrophages, human alveolar macrophages, and guinea pig macrophages (personal communication, R. Colvin, Massachusetts General Hospital, Boston).

When buffy coat leukocytes treated with A6F10 or its Fab fragments were analyzed by FACS, several observations were made. A majority of cells (85%) in the monocyte population, defined by its light-scattering properties, were intensely stained (Fig. 5a). In contrast to the microscopic methods used above, the more sensitive FACS technique revealed that neutrophils also bound A6F10, but at a level fourfold less than found on monocytes (Fig. 5b). A6F10 did not stain lymphocytes (Fig. 5c).

Cellular Specificity of A6F10. Since fibroblasts have been shown to carry plasma membrane receptors for fibronectin (26), we assessed the binding of A6F10 to two fibroblast cell lines (MRC-5 and FL-2000; Flow Laboratories, Inc., McLean, VA) and to primary human foreskin fibroblasts using fluorescence microscopic methods. Fluorescent staining was not observed in any of these cases.

A6F10 does not react with human umbilical cord endothelial cells in culture (personal communication, M. Bevilacqua, Harvard University, Brigham and Woman's Hospital, Boston). A6F10 reacts with guinea pig monocytes and macrophages but not with rat monocytes, mouse peritoneal macrophages, hamster monocytes, or the mouse macrophage cell line J774.

Cell Surface Antigen Recognized by A6F10. To identify the molecular species recognized by monoclonal antibody A6F10, NP-40 detergent lysates of human mononuclear cells were subjected to electrophoresis in SDS-PAGE, transferred to nitrocellulose filters by electroblotting, and incubated with A6F10 antibody

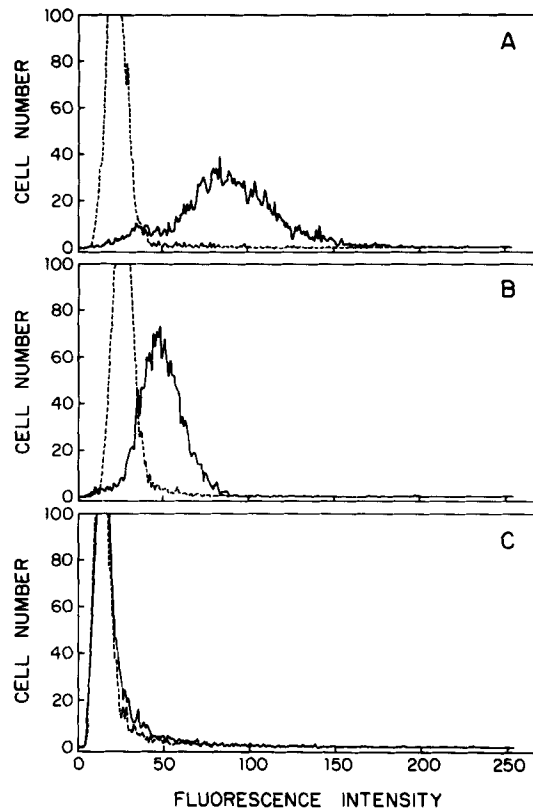


FIGURE 5. FACS analysis of A6F10 staining of blood leukocytes. A buffy coat preparation of human peripheral blood leukocytes was incubated with A6F10 ascitic fluid and stained with FITC-conjugated $F(ab')_2$ fragments of a goat antiserum to mouse IgG. Cell populations were analyzed by forward and right angle light scattering, and monocytes, neutrophils, and lymphocytes were gated for separate fluorescence analysis. Solid lines represent fluorescence intensity distribution of stained cells. Broken lines represent control cell preparations in which A6F10 was omitted. (a) Monocyte gated distribution. (b) Neutrophil distribution. (c) Lymphocyte distribution.

and then with $F(ab')_2$ fragments of a goat anti-mouse IgG labeled with ^{125}I . Fig. 6 shows that A6F10 specifically bound a protein with an apparent molecular weight of 110,000 (110 K). Ascitic fluid of mice bearing irrelevant hybridomas did not stain bands in this area of the gel (Fig. 6). The 110 K mol wt band showed the same mobility in reduced and nonreduced gels, indicating that it is made of a single polypeptide chain (Fig. 7).

The band observed with mononuclear leukocytes was also seen with human peripheral blood monocytes isolated by adherence to a plastic surface. Purified A6F10 IgG, as well as ascitic fluid from mice bearing five different subclones of A6F10, reacted with a species of the same apparent molecular weight. No reactive bands were observed in lysates of human erythrocytes, lymphocytes, or platelets.

Protease Susceptibility of the Antigen Recognized by Monoclonal Antibody A6F10. The interaction of monocytes with surfaces or particles coated with gelatin and fibronectin is abolished by pretreatment of the monocytes with trypsin (1). Thus,

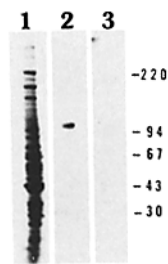


FIGURE 6. Western blot analysis of detergent lysates of human mononuclear leukocytes. The antigen to which the A6F10 antibody bound was determined by Western blot analysis. Lysate from 10^7 cells solubilized in 0.5% NP-40 was applied to each lane. (1) Coomassie Blue-stained gel of cell lysate. (2) Autoradiogram of Western blot with A6F10. (3) Autoradiogram of Western blot with irrelevant ascites. The positions of the standard proteins used to calibrate the gel are shown at right.

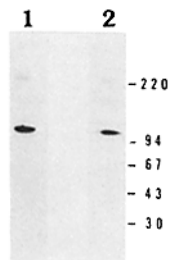


FIGURE 7. Western blot analysis of A6F10 reaction with reduced and nonreduced lysates of human mononuclear cells. Electrobots were prepared as in Fig. 6 (1) Reduced. (2) Nonreduced.

it was of interest to examine the susceptibility of A6F10 antigen to proteases. The 110 K mol wt antigen was resistant to endogenous proteases commonly present in cell preparations. Incubation of cells at 37°C in the absence of protease inhibitors for periods up to 60 min resulted in no decrease in the intensity of the 110 K band detected with A6F10 in Western blot analysis (Fig. 8, lanes 1 and 2). The antigen recognized by A6F10 was, however, susceptible to digestion by trypsin. Treatment of cells with $250\ \mu\text{g}$ trypsin resulted in complete loss of the 110 K mol wt band within 15 min of incubation at room temperature, conditions similar to those that abolish monocyte fibronectin receptor activity (Fig. 8, lanes 3–5). No lower molecular weight fragments reactive with A6F10 were observed. Similar results were observed using indirect immunofluorescence. Mononuclear cells attached to coverslips and incubated with $200\ \mu\text{g}$ trypsin for 25 min at 37°C , followed by $200\ \mu\text{g}$ trypsin inhibitor, showed substantial reduction of fluorescence staining by A6F10 compared with untreated controls. The proteolytic action of trypsin was confirmed by monitoring the loss of C3 receptor activity using the sheep erythrocyte rosette assay on coverslips prepared in parallel.

Discussion

Several lines of evidence indicate that monoclonal antibody A6F10 appears to recognize the plasma membrane receptor for fibronectin or a closely associated

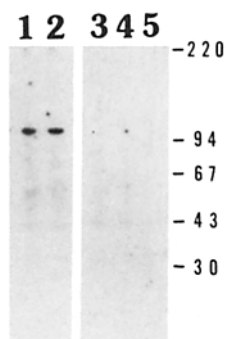


FIGURE 8. Proteolytic susceptibility of 110 K mol wt antigen. (1 and 2) Mononuclear cells (7.5×10^6) were incubated at 37°C in the absence of protease inhibitors for 0 (1) and 60 min (2) before NP-40 lysis and Western blot analysis with A6F10. (3, 4, and 5) Mononuclear cells (7.5×10^6) from the same preparation were incubated in DME containing $250 \mu\text{g}$ trypsin at room temperature for 15 (3), 30 (4), or 60 min (5). The reaction was terminated by addition of $250 \mu\text{g}$ soybean trypsin inhibitor. Cells were then washed, lysed with NP-40, and subjected to Western blot analysis with A6F10.

membrane structure of human peripheral blood monocytes. First, the fibronectin-mediated attachment of monocytes to gelatin-coated surfaces, which we previously described (1), is inhibited by A6F10. Fab fragments of the antibody retain this property, ruling out a possible interaction of A6F10 with membrane receptors for the Fc fragment of IgG, and indicating that the specificity is related to the antibody-combining region of the molecule. A6F10 also inhibits the binding of latex beads coated with gelatin and fibronectin to human peripheral blood monocytes. Monoclonal antibodies with undetermined specificities did not express this activity.

A6F10 did not interfere with other known receptors on the monocyte surface, particularly the receptors for Fc of IgG and for C3, in that it did not interfere with the attachment or phagocytosis of erythrocytes coated with these ligands. Also, the molecular species identified by A6F10 migrates with an apparent molecular weight of 110 K, different from those reported for the Fc receptor on neutrophils and monocytes (51–73 K) (27, 28) and for C3b receptors on monocytes and erythrocytes (205 K) (29, 30). The C3bi receptor consists of two polypeptide chains of 185 and 105 K mol wt (31). The smaller chain cannot be easily distinguished from the 110 K species we have obtained for the fibronectin receptor. In Western blot experiments (not shown) using monoclonal antibodies against the Fc receptor (3G8, kindly provided by Dr. J. Unkeless, The Rockefeller University) and to the C3b receptor (57F, kindly provided by V. Nussenzweig, New York University), both bands were clearly distinct from the 110 K mol wt fibronectin receptor.

The cellular distribution of the antigen recognized by A6F10 is also compatible with the cellular distribution of the fibronectin receptor on blood leukocytes. In immunofluorescence assays, the majority of peripheral blood monocytes stained intensely with A6F10. Neutrophils showed a low degree of fluorescence, detectable only by FACS analysis. Erythrocytes and lymphocytes did not stain with A6F10. The receptor for fibronectin has been previously recognized on mono-

cytes (1) and on neutrophils (32), and was not detected on erythrocytes or lymphocytes.

The protease sensitivity of the antigen recognized by A6F10 was also similar to that of the fibronectin receptor of monocytes. Trypsin treatment prevented monocyte attachment to fibronectin-gelatin surfaces and destroyed the antigen recognized by A6F10 in Western blots. Finally, the cell line U937 could be induced to express increased attachment to fibronectin-gelatin surfaces when exposed to gamma interferon. This is paralleled by increased expression of A6F10 antigen on the cell surface,² again supporting the association of the antigen recognized by A6F10 with the functionally recognized fibronectin receptors of monocytes. Proof of the specificity of the monoclonal antibody A6F10 will depend on the isolation of the plasma membrane antigen and the demonstration of its ability to specifically recognize fibronectin-gelatin surfaces and compete with monocytes in their attachment to these surfaces. These experiments are currently in progress.

The differences in intensity of staining between monocytes and neutrophils deserve special consideration. Neutrophils expressed at least fourfold less A6F10 antigen than monocytes. Also, a population of ~15% monocytes showed the same staining intensity as neutrophils. In five experiments, FACS analysis showed that 72–96% (\bar{x} = 85%) of monocytes possess detectable A6F10 antigen. In the cell attachment assays, ~85% of the peripheral blood monocytes bound effectively to plates coated with gelatin and 40 $\mu\text{g}/\text{cm}^2$ fibronectin (unpublished observation). 20% of the circulating monocytes identified by nonspecific esterase staining were unable to attach to these surfaces. On the other hand, neutrophils do not attach to fibronectin-gelatin surfaces (1). These observations suggest that 20% of the monocytes and 100% of the neutrophils in circulation have fewer fibronectin receptors than required for effective attachment to fibronectin-coated surfaces. In essence, fibronectin receptors seem to mediate at least two different functions: they induce functional responses such as the expression of increased Fc and C3 receptor activity, and mediate attachment of monocytes to surfaces containing fibronectin, expressed only by those monocytes with a higher density of plasma membrane receptors.

Cells other than monocytes, most notably fibroblasts, bind fibronectin (26). Our finding that the A6F10 monoclonal antibody does not bind to fibroblasts indicates that the monocyte receptor contains antigenic determinants distinct from those of fibroblast receptors for fibronectin. Other differences between monocyte and fibroblast receptors have been recognized: monocytes, unlike fibroblasts, do not synthesize fibronectin and do not contain fibronectin on their surfaces. Fibronectin is detected on macrophages only during long-term culture (33). Fibroblasts, on the other hand, contain fibronectin in their pericellular matrix (34). The receptors of monocytes and fibroblasts have different protease sensitivity profiles and Mg^{++} requirements. The monocyte fibronectin receptor is trypsin sensitive and requires Mg^{++} for its activity (1), whereas the fibroblast receptor is not trypsin sensitive (35) and does not require Mg^{++} for binding (26). However, these differences do not preclude the existence of structural and

² Hosein, B., A. Brock, and C. Bianco. Manuscript in preparation.

functional similarities between fibronectin receptors of fibroblasts and macrophages.

The 110 K mol wt surface protein to which A6F10 binds is substantially smaller in molecular weight than the 140 K fibronectin receptor recently isolated from a fibroblast cell line (36). However, antibodies that block the binding of fibroblasts to fibronectin-coated surfaces immunoprecipitate a complex of proteins of 160–95 K mol wt, of which the 140 K receptor appears to be a member. It is possible that the 110 K mol wt protein of monocytes may associate with proteins analogous to the 140 K species. A smaller fibroblast surface protein of 48–49 K mol wt has also been proposed as the fibroblast fibronectin receptor. It can be crosslinked to substrate-bound fibronectin (37) and has been proposed to be involved in cell adhesion to fibronectin, based on its calcium-dependent stability to trypsin and its binding to wheat germ agglutinin (35). Pytela et al. (36) have suggested that this protein may be involved in the assembly of fibronectin into matrix (38) whereas the 140 K mol wt protein is involved in cell attachment.

Because of its specificity for mononuclear phagocytes, the monoclonal antibody A6F10 provides a specific probe for cells of monocyte lineage in tissues in vitro, and in marrow populations. It may also prove to be a valuable tool in assessing the role of fibronectin in monocyte function.

Summary

We describe a molecule on the surface of human peripheral blood monocytes that appears to be a plasma membrane receptor for fibronectin. We have identified this protein using a monoclonal antibody, A6F10, which prevents the interaction between monocytes and substrate-bound fibronectin. Thus, at least functionally, the antibody appears to recognize the plasma membrane receptor for fibronectin. The antibody and its Fab fragments bound to the cell surfaces of human monocytes, tissue macrophages, and, to a lesser extent, neutrophils. It did not react with fibroblasts, lymphocytes, platelets, or erythrocytes. It bound human and guinea pig cells but did not react with rat, mouse, or hamster cells. In Western blots, this monoclonal antibody bound specifically to a polypeptide with apparent molecular weight of 110,000 and made of a single chain. The antigen recognized by A6F10 was susceptible to trypsin digestion. These observations suggest that the monoclonal antibody A6F10 is directed to the fibronectin receptor of human monocytes.

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