Lymphokines and Platelets Promote Human Monocyte Adherence to Fibrinogen and Fibronectin In Vitro

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Monocytes must accumulate in areas of tissue injury and inflammation to effect phagocytosis, antigen presentation, and monokine production. Fibrinogen/fibronectin matrices have been demonstrated in healing wounds and in delayed-type hypersensitivity skin reactions. We have developed an in vitro system for investigation of the ability of fibrinogen and fibronectin matrices to serve as substrata for human peripheral blood monocyte adherence. Monocyte adherence was greatest on matrices of both fibrinogen and fibronectin, less to fibrinogen alone, and least to fibronectin alone. Lymphokines increased adherence of monocytes to all three surfaces but not to albumin-coated surfaces.

Addition of platelets also caused a dose-dependent increase in monocyte adherence to all three surfaces. This increased adherence was not a simple function of arachidonic acid metabolites, stable platelet products, nor monocyte binding sites on the platelet membrane. The effect of platelets was not additive to the effect of lymphokines.

We conclude that fibrinogen and fibronectin provide a framework for monocyte adherence and that factors present in areas of inflammation and wound healing, such as lymphokines and platelets, can augment this adherence. Such adherence facilitates the transformation of monocytes into macrophages in vitro and may also foster such transformation in vivo.

Key words: lymphokines, platelets, monocyte, adherence, fibrinogen, fibronectin

INTRODUCTION

Monocyte/macrophage accumulation in areas of inflammation or tissue injury is important for tissue debridement through phagocytosis, stimulation of the immune response through antigen presentation and interleukin-1 production [3,27,42], and initiation of wound repair through release of endothelial cell and fibroblast growth factors [9,10]. Localization of peripheral blood monocytes to a specific tissue site is the manifestation of both chemoattraction and retention. Cell retention at a tissue site could result from increased cell adhesiveness or increased substrate adhesiveness. As in vitro examples, macrophage migration inhibition factor (MIF) acts by a cellular mechanism to increase cell adhesiveness [20], while fibronectin alters the substrate to increase cell adhesion [43]. Evidence that these factors function in vivo is provided by Nelson's classic experiments on peritoneal macrophage disappearance [36] and by the observation that macrophages localized in a delayed hypersensitivity reaction stain for fibronectin by immunofluorescence techniques [14], while peripheral blood monocytes do not [1].

Platelets also promote monocyte adherence in vitro [35] and may do the same in vivo. They have been shown to release thromboxane and platelet-derived growth factor (PDGF), factors that activate neutrophils [22,44] and could lead to increased adherence by a cellular mechanism. Platelets also express fibronectin on their surface [25,26], and this might cause increased monocyte adherence by providing a substrate for cell attachment.

In this report we confirm that fibrinogen and fibronectin provide an excellent matrix for the adherence of human peripheral blood monocytes. Both platelets and lymphokines augment monocyte adherence to these matrices. These observations provide a model for understanding how monocytes localize in vivo.

METHODS

Blood Donors

Blood was obtained from healthy volunteers. Informed consent was obtained and the guidelines of the Institutional Review Board of the National Jewish Center for Immunology and Respiratory Medicine were observed.

Separation of Mononuclear Cells

Twenty milliliters of citrated (0.38%) blood was obtained from each donor. To avoid significant platelet
contamination, platelet-rich plasma (PRP) was removed by centrifugation at 300g for 20 min at room temperature as recommended by Musson and Henson [35]. After the PRP was removed the cells were resuspended in a volume of Hanks' balanced salt solution (HBSS) equal to the amount of PRP removed. The cell suspension was then layered over 5 cc of Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) in 15-ml centrifuge tubes and centrifuged for 25 min at 750g at room temperature. The mononuclear cell suspension was carefully removed from the Ficoll-Hypaque interface, washed three times in iced HBSS without calcium or magnesium at 4°C, and then resuspended in RPMI 1640 for use in monocyte adherence assays or preparing lymphokine containing supernatants. All preparations contained a platelet:mononuclear cell ratio of 3:1 or less.

Preparation of Monocyte Monolayers

Mononuclear cells were separated as described and suspended at a concentration of 5 x 10⁶ cells per ml in RPMI 1640 (GIBCO, Grand Island, NY) with 10 μg/ml streptomycin and 100 units/ml penicillin (GIBCO, Grand Island, NY). Two hundred microliters of cell suspension were placed into each well of a 96-well flat-bottomed tissue culture plate (Flow Laboratories, Inc., No. 76-003-05, McLean, VA) and incubated for 60 min at 37°C in a 5% CO₂-95% water-saturated air mixture [40]. Non-adherent cells were removed by washing each well four times with 300 μl of room-temperature phosphate-buffered saline (PBS). PBS was delivered onto the side wall of the well through an 18-gauge sterile needle. A constant and reproducible flow of washing fluid onto the monolayers was ensured by use of an apparatus designed to deliver PBS by gravity feed from a reservoir with a pressure that could be varied from 1 to 45 cm [33]. Washes were removed by aspiration of the corner of the well with a sterile 23-gauge needle. Monolayers were stained for nonspecific esterase as previously described [46], and in every experiment the monolayers contained >90% esterase-positive cells. Because of donor variability, the actual number of adherent monocytes varied from experiment to experiment. Each figure shows experiments done with a preparation from one donor; however, numerical comparisons between figures cannot be made since these experiments were done on separate days with different donors.

Enumeration of Adherent Monocytes

To evaluate adherence of monocytes to surfaces over time periods from 15 to 120 min, mononuclear cells were labelled with ⁵¹Cr before being applied to the wells [45]. In all, 5 x 10⁷ cells/ml were suspended in RPMI 1640 with 200 μCi/ml of Na⁵¹CrO₄ and incubated in a shaking water bath for 45 min at 37°C. Unbound ⁵¹Cr was removed by washing the suspension in iced HBSS without calcium or magnesium. Cells were then resuspended in RPMI 1640 for application to the coated surfaces. To evaluate adherence of monocytes to surfaces over time periods from 3 to 72 h adherent cells were labelled in situ at the end of the time period. Each well of adherent cells was aspirated and then covered with 50 μl of a solution of 2 μCi/ml Na⁵¹CrO₄ (New England Nuclear, Boston, MA) in RPMI 1640. After 3 h the wells were washed four times as described above. Total cell-associated radioactivity was measured by adding 50 μl of 1% sodium dodecyl sulfate (SDS) to each well and removing the resulting suspension with a cotton-tipped swab. Samples were counted in a Beckman Gamma 8000 (Beckman Instruments Inc, Fullerton, CA). This method has previously been shown to correlate closely with direct counting of monocytes [33]. The small amount of ⁵¹Cr absorbed by platelets contaminating the mononuclear cell preparations did not significantly alter the results.

Preparation of Lymphokine-Containing and Control Supernatants

Mononuclear cells from Hypaque-Ficoll gradients were suspended at a concentration of 10⁷ cells per ml in RPMI 1640 with streptomycin and penicillin in borosilicate glass test tubes. Then 32/8 units/ml of streptokinase-streptodornase (SK-SD; Varidase; Lederle Laboratories, Pearl River, NY) was added to cell suspensions to produce lymphokine-containing supernatants; the control supernatants received no antigen. All tubes were incubated at 37°C in 5% CO₂-95% water-saturated air. At 48 h tubes were centrifuged for 15 min at 1,000g and supernatants were harvested. Then 32/8 units/ml SK-SD was added to control supernatants. Supernatants were reconstituted with glucose, amino acids for minimal essential medium (MEM), and nonessential amino acids for minimal essential medium (Flow Laboratories, Inc, McLean, VA), and the pH was adjusted with bicarbonate to 7.2-7.4.

Assay of Lymphokine-Induced Adherence

Adherence was assayed using a modification of the method of Rocklin et al [41]. Indicator monocyte monolayers were prepared in quadruplicate as described herein and covered with 200 μl of either control or lymphokine-containing supernatant. Wells were incubated for 72 h, then washed and labelled with ⁵¹Cr as described above.

Fibronectin Preparation

Fibronectin was purified by the gelatin-Sepharose affinity chromatography method [24]. Because substantial amounts of fibronectin degradation products were recovered in the eluate from the gelatin-Sepharose column, the procedure was modified to yield intact fibronectin.
The original affinity purification procedure and subsequent modifications are outlined below.

To produce unfragmented fibronectin, we collected blood in $10^{-3}$ M phenylmethylsulfonyl fluoride (PMSF) and included this serine protease inhibitor in all buffers during gelatin-affinity chromatography and subsequent ion exchange and molecular sieve chromatography. The eluate from gelatin-Sepharose was dialyzed against 0.05 M NaCl, 0.01 M Tris (pH 8.0), and was applied to a 50-ml bed volume diethylaminoethyl (DEAE) cellulose (DE-52, Whatman, Clifton, NJ) column equilibrated in the same buffer. After washing the column with three bed volumes of the buffer, fibronectin was eluted with 500 ml of a linear gradient of 0.05 M NaCl to 0.3 M NaCl in 0.01 M Tris (pH 8.1). A small peak was removed during the wash and a second small peak was eluted shortly after the application of the salt gradient. These peaks were pooled, concentrated, and analyzed by Ouchterlony immunodiffusion, which showed that the first peak was IgG and the second peak was fibronectin. A large peak of fibronectin was eluted with higher salt concentrations. Fractions containing fibronectin were pooled, concentrated by negative pressure dialysis, dialyzed against PBS (pH 7.4) and applied to a Sepharose 4B column (Pharmacia Fine Chemicals, Piscataway, NJ), which yielded a large single peak of fibronectin. By SDS-gradient polyacrylamide gel electrophoresis (PAGE), immunoelectrophoresis, and double immunodiffusion, the concentrated fibronectin preparation was unfragmented [8] and was not contaminated with detectable amounts of immunoglobulins, albumin, fibrinogen, or other serum proteins.

**Preparation of Human Fibrinogen**

Fresh platelet poor plasma, anticoagulated by 0.01 M edetate (EDTA), was passed through lysine-Sepharose [23] and gelatin-Sepharose affinity [24] columns sequentially to adsorb plasminogen and fibronectin from the plasma, respectively. Fibrinogen (Cohn I-1) was isolated from the plasma effluent by the method of Blomback and Blomback [6]. The final fibrinogen precipitate was redissolved in distilled water and dialyzed extensively against 0.05 M NaCl, 0.01 M Tris buffer (pH 8.0) for ion exchange chromatography on a DEAE cellulose (Whatman Ltd, Clifton, NJ) column equilibrated with the same buffer. Fibrinogen was eluted by a linear salt gradient from 0.05 M NaCl to 0.3 M NaCl in Tris buffer (pH 8.0) and separated from small amounts of contaminant IgG and albumin. The final preparation gave no precipitation line on double immunodiffusion against rabbit antihuman serum, antihuman IgG, and antihuman albumin (Cappel, Cochraville, PA) or antihuman fibrinogen. A single precipitin band was seen when run against antihuman fibrinogen (Cappel, Cochraville, PA).

**Preparation of Protein-Coated Surfaces**

Protein-coated wells for adherence assays were prepared by adding 50 μl of a protein solution (see above) to tissue culture, 6-mm-diameter, flat-bottom microtiter wells (Linbro Scientific, Hamden, CT) for 2 h at 22°C [31,38]. Since some plastic protein binding sites may still be available after application of the first protein coat, we added 50 μl of a 20 mg/ml human serum albumin (HSA) (Calbiochem, La Jolla, CA) solution to each well for 2 h at 22°C to block these sites [28]. In some experiments, a final protein solution of fibronectin was added to the plate by incubating with a 50-μl aliquot of a fibronectin solution for 2 h at 22°C. Since plastic protein binding sites were blocked by HSA, these final proteins must interact with first coat proteins to bind to the wells. Wells were washed with PBS three times between coagings. In some experiments 5 U/ml thrombin was included with the 100 μg/ml fibronectin that was added to wells that had previously been coated with fibronectin to convert the fibronectin to fibrin. This could have also led to fibronectin-fibrinogen cross-linking by factor XIII known to contaminate fibrinogen preparations.

**Quantitation of Fibrinectin Adsorption Onto Microtiter Wells**

We investigated the amount of fibronectin adsorbed onto 6-mm-diameter, flat-bottom microtiter wells that were either uncoated or previously coated with fibronectin (see above). Fibronectin was radiolabeled with $^{125}$I-Bolton Hunter reagent (New England Nuclear, Cambridge, MA) as previously described [7]. Free and protein-bound $^{125}$I-reagent were separated by gel filtration over a Sephadex G-25 column (Pharmacia Fine Chemicals, Piscataway, NJ). Final fibronectin specific radioactivity was 26.8 μCi/mg. Solutions of 300, 150, 75, 38, and 19 μg/ml $^{125}$I-fibronectin were added to microtiter wells in 50-μl aliquots, incubated 2 hours at 22°C, and washed three times with PBS. Microtiter wells were cut from the plate with a miniature, carbon-steel circular power saw and counted in a Beckman Gamma counter.

**Platelet Preparation**

Platelets were isolated from fresh citrate-phosphate-dextrose human blood by centrifuging at 250g for 20 min, then spinning the recovered platelet-rich plasma (PRP) at 2,200g for 15 min. The platelet pellet was washed three times in 150 mM NaCl, 3 mM NaHPO$_4$, 8 mM KCl, 5.5 mM glucose (modified PBS), pH 6.5, containing 100 μg/ml apyrase (Sigma, St Louis, MO), resuspended to a final concentration of 2.5 × 10$^7$ platelets/ml in Hanks' MEM, pH 7.3, containing 4 mg/ml human serum albumin. In experiments where platelet:mononuclear cell concentrations were evaluated, autologous mononuclear cells were added to a suspension
of platelets in RPMI and the suspension was immediately added to the microtiter cells.

**Release of $^3$H-Serotonin From Platelets**

In order to monitor platelet release reactions, human platelet preparations were labeled with $^3$H-serotonin as previously described [2], and $^3$H-serotonin release was measured after addition of thrombin. Experiments were performed in a model DP-247-D dual sample aggrengometer (Scienco, Morrison, CO). The standard reaction mixture consisted of 250,000 platelets/µl in a total volume of 600 µl of modified Tyrode's solution (NaCl, 8 g/liter; KCl, 195 mg/liter; NaHCO₃, 1 g/liter; MgCl₂⋅6H₂O, 213 mg/liter; D-glucose, 1 g/liter; gelatin, 5 g/liter; CaCl₂, 145 mg/liter; adjusted to pH 7.2). The platelets were continuously stirred at 1,200 rpm and warmed to 37°C in siliconized glass cuvettes. Before stimulation, a 100-µl aliquot of the platelet suspension was removed to determine background $^3$H-serotonin levels for that particular cuvette. This was accomplished by pelleting the 100-µl aliquot of platelets at 12,000g for 60 s in a model 3200 Brinkman centrifuge. Fifty microliters of the supernatant was mixed with 5 ml of Aquasol (New England Nuclear, Boston, MA) and counted in a LS8000 liquid scintillation counter (Beckman Instruments, Irvine, CA). The remaining 500 µl of platelet suspension was stimulated with 5 U human α-thrombin, and the increase in light transmission through the platelet suspension, indicating aggregation, was recorded on a chart recorder. Sixty seconds after stimulation, a 100-µl aliquot of the platelet suspension was assayed for $^3$H-serotonin release as described above. The amount of $^3$H-serotonin contained in the platelet suspension was determined by lysing 500 µl of unstimulated platelets with 50 µl of 2.5% Triton X-100 and assaying for $^3$H-serotonin in a 100-µl aliquot of the detergent-lysed platelets as described above. The amount of $^3$H-serotonin contained in the platelets was used to calculate the percent secretion of $^3$H-serotonin induced by thrombin as previously described [19]. Human platelet preparations always gave between 80–95% serotonin release upon α-thrombin stimulation and a brisk, complete aggregation response.

**Preparation of Fixed Platelet Monolayers**

A total of $20 \times 10^6$ isolated platelets were added to each microtiter well, incubated for 30 min at 37°C, and then either stimulated with 1 U/ml thrombin for 10 min at 37°C or left unstimulated. Both unstimulated and stimulated platelet monolayers were fixed with formalin 2% in PBS for 10 min, then washed three times prior to the addition of monocytes. The presence of a monolayer of platelets adherent to the well was confirmed by examination with an inverted microscope.

**Preparation of Platelet Release Products**

Isolated platelets ($2.5 \times 10^9$/ml in RPMI) were stimulated with 1 U/ml human α-thrombin (Sigma, St Louis, MO) for 10 min at 37°C and then centrifuged at 1,000g for 15 min. This supernatant was designated "platelet release products" (PREP). A 200-µl suspension of $5 \times 10^6$ mononuclear cells/ml in PREP was then placed in each well of a 96-well flat-bottomed tissue culture plate and incubated for 60 min at 37°C in a 5% CO₂-95% water-saturated air mixture. Control wells were prepared with $5 \times 10^6$ mononuclear cells/ml in RPMI. Nonadherent cells were removed by washing as described above. The adherent monolayers were then covered with PREP or RPMI alone (controls) and incubated for 72 h.

**Inhibition of Arachidonic Acid Metabolite**

The effect of arachidonic acid metabolites on monocyte adherence was assayed using indomethacin and nordihydroguaiaretic acid (NDGA). A suspension of mononuclear cells in RPMI was prepared which contained either a 10:1 or a 20:1 platelet:mononuclear cell ratio. Each of these was then adjusted to a final concentration of $5 \times 10^6$ mononuclear cells/ml and either $3 \times 10^{-6}$ M indomethacin or $3 \times 10^{-6}$ M NDGA. Quadruplicate 200-µl aliquots of each suspension were added to wells of a microtiter plate, incubated for 1 h, and washed as described above. Adherent cells were then covered with RPMI, RPMI with $3 \times 10^{-6}$ M indomethacin, or RPMI with $3 \times 10^{-6}$ M NDGA and incubated for 72 h.

**RESULTS**

**Fibrinogen and Fibronectin Binding to Plastic Surfaces**

We coated surfaces with fibrinogen, fibronectin, and fibrinogen/fibronectin to assess monocyte binding to these proteins in vitro. To determine whether maximal adsorption onto the surface had occurred, we applied radiolabelled fibronectin to the uncoated plastic surface and to surfaces that had previously been coated with fibrinogen at concentrations of 100 or 1,000 µg/ml. Concentrations of fibronectin were added to the plates in the range of human serum fibronectin concentrations (20–300 µg/ml). As shown in Figure 1, maximal fibronectin-plastic binding occurred above 75 µg/ml. Fibronectin binding to fibrinogen was relatively constant over the range of fibrinogen tested; no additional fibronectin was bound when the fibrinogen coat was applied at the higher concentration. Addition of 5 U/ml thrombin to fibronectin solutions did not result in additional incorporation of fibronectin onto the fibrinogen (fibrin) coat.
Time Course of Monocyte Attachment to Coated Surfaces

Increasing numbers of cells attached to coated surfaces over the first 60 min of incubation (Fig. 2). Slightly fewer cells remained attached if the incubation was extended to 120 min. Therefore, in succeeding experiments 60 min was allowed for monocyte attachment.

Relation of Monocyte Attachment to Amount of Surface-Bound Protein

As seen in Figure 3, the number of monocytes that attached to the surface increased as the concentration of protein applied to the well increased from 10 to 100 µg/ml. Higher concentrations of fibrogen actually resulted in decreased monocyte attachment. Therefore, in all subsequent experiments both fibrogen and fibronectin were applied at concentrations of 100 µg/ml. Saturation of plates with albumin resulted in minimal nonspecific binding. Moreover, saturation of plates with albumin after coating with fibrogen or fibronectin had no effect on monocyte attachment.

Monocyte Adherence to Protein-Coated Surfaces

Figure 4 shows the relative monocyte adherence to the protein surfaces and its decline over a 72-h period. Fibrogen plus fibronectin yielded slightly better mono...
cyte adherence than fibrinogen alone, while fibronectin alone resulted in significantly less adherence. When 5 U/ml thrombin and 100 μg/ml fibronectin were added simultaneously to fibrinogen-coated surfaces to generate fibronectin-fibrin substrata, monocyte adherence to these substrata was no greater than to fibronectin-fibrinogen substrata. Adherence to fibronectin was no better than adherence to uncoated tissue culture plastic (Fig. 4) or to plastic coated with human serum albumin (data not shown).

**Effect of Lymphokines on Monocyte Adherence to Protein-Coated Surfaces**

We and others have previously shown that lymphokine-containing supernatants can induce increased monocyte adherence to tissue culture plastic over a 72-h incubation [33,41]. We therefore assessed the ability of lymphokines to induce increased monocyte adherence to protein-coated surfaces. As shown in Figure 5A, lymphokine-induced increase in adherence was observed on fibronectin, fibrinogen, and fibrinogen/fibronectin-coated surfaces. No lymphokine effect was seen on plastic coated with human serum albumin (Fig. 5B).

**Fig. 4.** Time course of human peripheral blood monocyte adherence to plastic and coated surfaces. A 200-μl aliquot of a suspension of 5 x 10⁶ mononuclear cells/ml was added to each well. Each point represents the mean ± SEM of four replicate experiments.

**Fig. 5.** Monocyte adherence to protein-coated surfaces modulated by lymphokines. Cells incubated with control supernatants are indicated by open bars; cells incubated with lymphokine-containing supernatants are indicated by hatched bars. FN = fibronectin; FG = fibrinogen. A: Lymphokine-incubated monocytes showed increased adherence on all three surfaces. B: No increase was seen on albumin-coated surface. Each bar represents the mean ± SEM of four replicate experiments.
Effect of Platelets on Monocyte Adherence to Protein-Coated Surfaces

Platelets have previously been shown to affect human monocyte adherence [35]. Our mononuclear cell preparations were relatively platelet-free (less than 3:1 platelet:cell ratio). Therefore we added platelets to cell preparations in ratios equivalent to those attained in vivo and incubated them for 72 h on protein-coated surfaces. The results are shown in Figure 6. Higher ratios yielded markedly increased monocyte adherence to fibrinogen and fibrinogen/fibronectin and moderately increased adherence to fibronectin alone.

The effect of platelet release products on monocyte adherence is illustrated in Table 1. Platelets were stimulated with thrombin and platelet release products were collected. Adequacy of platelet stimulation was confirmed by ³H-serotonin release (see Materials and Methods). These release products were then added to monocyte monolayers to determine their effect on adherence. As can be seen in Table 1, this resulted in a diminution of monocyte adherence.

In order to evaluate whether platelet membranes provide a framework for monocyte adherence, we prepared platelet monolayers on protein-coated surfaces. These monolayers were either stimulated with 1 U/ml thrombin, or left unstimulated, and then fixed in formalin, or not fixed. Monocyte adherence was not augmented on stimulated platelet monolayers (Table 1). Fixation actually diminished monocyte adherence to platelet monolayers.

The effect of arachidonic acid pathway inhibitors on monocyte adherence was evaluated by adding either indomethacin (3 × 10⁻⁶ M) or nordihydroguaiaretic acid (NDGA) (3 × 10⁻⁶ M) to the platelet:mononuclear cell preparation. These concentrations have previously been shown to affect both platelet and neutrophil function [4,44]. Neither indomethacin nor NDGA prevented the platelet-induced increased monocyte adherence (data not shown).

Simultaneous Stimulation of Monocyte Adherence With Platelets and Lymphokines

Monocyte adherence to protein-coated surfaces appears maximally stimulated with lymphokine supernatants and with high platelet:mononuclear cell ratios (Fig. 7). Thus, lymphokines cannot further enhance monocyte adherence when the monocytes are plated in the presence of large numbers of platelets. Likewise, platelets cannot further stimulate monocyte adherence in the presence of lymphokines.

DISCUSSION

We have investigated the ability of lymphokines and platelets to enhance human peripheral blood monocyte adherence to protein substrata composed of fibrinogen and fibronectin. Both lymphokines and platelets were found to enhance monocyte adherence in vitro. Fibrinogen and fibronectin matrices have been demonstrated in vivo both in wound healing [11,13] and in delayed-type hypersensitivity (DTH) reactions [12,14,16–18], where they are thought to provide a framework for monocyte adherence. Our results support this hypothesis and suggest that lymphokines and platelets might enhance such adherence in vivo.

Fibrinogen, fibronectin, and fibrinogen/fibronectin substrates were prepared by adsorbing protein solutions onto tissue culture plastic in microtiter wells. It has previously been shown that fibronectin adsorption to tissue culture plastic produces a more favorable molecular orientation for cell binding than adsorption to other surfaces [28,29]. Figure 1 demonstrates the relationship between protein concentration added and protein bound to plastic. In other experiments a final coat of albumin...
TABLE 1. Effect of Platelet Release Products and Fixed Platelet Monolayers on 72-Hour Monocyte Adherence

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Protein coats</th>
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<tbody>
<tr>
<td></td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>RPMIa</td>
<td>2,563 ± 404b</td>
</tr>
<tr>
<td>RPMI with 20 × 10⁶ platelets</td>
<td>5,809 ± 321</td>
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<tr>
<td>RPMI with platelet release productsd</td>
<td>(+127%)c</td>
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<tr>
<td>Viable stimulated platelet monolayersd</td>
<td>1,099 ± 138</td>
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<tr>
<td>Fixed stimulated platelet monolayersd</td>
<td>2,754 ± 246</td>
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<tr>
<td>Fixed unstimulated platelet monolayersd</td>
<td>920 ± 155</td>
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aBaseline monocyte adherence in the 3-day post-labeling assay with a platelet:mononuclear cell ratio of 2:1.
bMean ± SEM of four replicate wells of adherent monocytes expressed in counts/min.
cNumber in parentheses represents percent change from medium alone.

was added to occupy any potentially available plastic sites available for cell binding. Monocyte adherence was not affected by this addition. Investigation of the kinetics of monocyte attachment to protein-coated surfaces revealed that maximal attachment occurred at 60 minutes (Fig. 2), when surfaces were coated with proteins applied at a concentration of 100 μg/ml (Fig. 3). These attachment kinetics are similar to those reported for monocyte attachment to plastic alone [41] and other coated surfaces [5].

Lymphokine effects on human monocyte adherence in culture are maximal at 72 h [41]. We therefore observed the adherence characteristics of monocytes over a 3-day period. The kinetics are similar to those seen when cells are adherent to tissue culture plastic [33,41]. A fibrinogen/fibronectin matrix enabled the most cells to remain adherent (Fig. 4) mainly by promoting maximal attachment within the first hour (Fig. 2). Fibrinogen alone was slightly less effective but enabled significantly more cells to remain adherent than either fibronectin alone or plastic. It is interesting to note that fibronectin alone was not nearly as effective as fibrinogen/fibronectin (Fig. 4), despite the fact that three times as much fibronectin bound to plastic as bound to fibrinogen (Fig. 1). Fibronectin binds to fibronectin only at specific sites [43], while binding to plastic may be nonspecific. This further supports the observation of Grinnell et al [30] that fibronectin orientation rather than the amount of fibronectin determines cell binding efficiency.

Lymphokines have been shown to enhance human monocyte adherence to plastic [33,41]. In animal studies macrophages rendered immobile by MIF deposit a fibrin matrix onto plastic [34], while macrophages in a DTH reaction possess significantly greater amounts of fibrinogen and fibronectin on their surface than do control cells [15,32]. In the guinea pig fibronectin has been reported to enhance the response of macrophages to MIF [39]. Human monocytes possess fibronectin receptors [5,37] and secrete fibronectin into culture medium [1]. More-
over, we have recently shown that human macrophages in a DTH reaction stain for fibronectin in vivo [14]. Therefore we examined the ability of lymphokine preparations to enhance monocyte adherence to matrices composed of fibrinogen and fibronectin.

A lymphokine effect was seen on all three matrices (Fig. 5) but not on albumin-coated plastic, indicating that there is some substratum specificity. The magnitude of the lymphokine-mediated increase in adherence was approximately equal on all three surfaces and equivalent to lymphokine-induced increases seen on plastic [33,41]. The fibrinogen/fibronectin substratum consistently enabled more cells to remain adherent than either fibrinogen or fibronectin alone. These results suggest that fibrin/fibrinogen-fibronectin matrices may function in vivo as the framework for lymphokine-induced monocyte adherence in areas of immunologically mediated inflammation.

The lymphokine responsible for increased monocyte adherence has not been defined. Lymphokine-rich supernatants such as those employed in these experiments have been shown to contain MIF activity; column fractionation revealed that the factor responsible for this activity co-eluted with chymotrypsinogen, molecular weight 23,000 [41]. However, this fraction would also contain other lymphokines, such as gamma interferon (IFN-γ), known to activate monocytes. Recent investigations suggest that MIF and IFN-γ are identical or closely related compounds [21]. Other soluble mediators may also be involved.

Areas of nonimmunologic inflammation, such as those of wound healing, also have prominent deposition of fibrin and fibronectin [11,13] and a need for monocyte localization to promote phagocytosis of tissue debris. Platelets are often seen in concert with fibrin in such areas and have been reported to increase monocyte adherence to plastic in vitro [35]. We investigated the ability of platelets to promote monocyte adherence to fibrinogen and fibronectin. As shown in Figure 6, the addition of platelets led to increased adherence on all three surfaces.

There are several possible explanations for this phenomenon. First, thrombin-stimulated platelets express fibronectin on their surfaces [25,26], suggesting that they may provide an additional surface for monocyte adherence. We investigated this possibility by preparing formalin-fixed platelet monolayers and evaluating monocyte adherence to the monolayers. These were not nearly as effective as control wells, perhaps because the fixed platelets obstructed monocyte access to the binding surface. We therefore feel it is unlikely that platelets increase monocyte adherence simply by providing a framework of fibronectin or other proteins for monocyte binding.

Second, stimulated platelets secrete fibronectin [25,26,47] as well as PDGF and thromboxanes, which might then stimulate monocytes to adhere. We therefore prepared supernatants containing the soluble products released by thrombin-stimulated platelets. These supernatants did not cause increased monocyte adherence (Table 1). However, since these supernatants were prepared separately and later added to cell suspensions, soluble mediators with a short duration of action such as thromboxane or other arachidonic acid metabolites would not be detected in such an assay. In order to assess the importance of cyclooxygenase and lipoxygenase pathway products in the increased monocyte adherence induced by platelets we evaluated the increased adherence in the presence of indomethacin and NDGA. However, increased adherence was not affected under these conditions. Our current hypothesis is that short-acting or labile platelet-derived mediators other than those of the arachidonic acid pathway may be responsible for the increased monocyte adherence. Alternatively, synergy between several factors or an interactive process between the platelets and monocytes might be responsible for the platelet-induced increase in monocyte adhesion. These phenomena would not be detected by the individual assays that we employed.

Monocytes stimulated by platelets may have achieved maximal “activation” since they are unable to be further activated by lymphokines. In the experiment shown in Figure 7, platelets were first added to cell suspensions; then the monolayers were washed and control or lymphokine containing supernatants added for a 72-h incubation. The addition of platelets abrogated the lymphokine effect by raising adherence of control cultures to the level of lymphokine-stimulated cultures. Platelets had no effect on lymphokine-stimulated cells. This suggests that platelets activate monocytes to adhere in a similar way and to a similar degree as do lymphokines. There may, in fact, be a common pathway for monocyte activation that operates in both cell-mediated and traumatic inflammation.

In the first case the pathway is activated by antigens and T-cell-derived factors, while in the second case platelets provide the activating signal. As we have shown, the activation in either case is maximal and not additive.

These studies provide support for the pivotal role of fibrinogen and fibronectin in maintaining peripheral blood monocytes in areas of tissue inflammation and repair. Once attracted to such an area by chemotactic factors, a monocyte must persist for long enough to undergo transformation into a macrophage. The investigations reported here show that both lymphokines and platelet-dependent factors can induce monocytes to adhere to matrices of fibrinogen, fibronectin, or both for a period sufficient to effect this transformation.
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REFERENCES

35. Nelson, D.S. The effects of anticoagulants and other drugs on cellular and cutaneous reactions to antigen in guinea-pigs with
Horsburgh, Clark, and Kirkpatrick