Fibrin Regulates Neutrophil Migration in Response to Interleukin 8, Leukotriene B4, Tumor Necrosis Factor, and Formyl-Methionyl-Leucyl-Phenylalanine

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Summary

We have examined the capacity of four different chemoattractants/cytokines to promote directed migration of polymorphonuclear leukocytes (PMN) through three-dimensional gels composed of extracellular matrix proteins. About 20% of PMN migrated through fibrin gels and plasma clots in response to a gradient of interleukin 8 (IL-8) or leukotriene B4 (LTB4). In contrast, <0.3% of PMN migrated through fibrin gels in response to a gradient of tumor necrosis factor α (TNF) or formyl-methionyl-leucyl-phenylalanine (FMLP). All four chemoattractants stimulated PMN to migrate through gels composed of collagen IV or of basement membrane proteins (Matrigel), or through filters to which fibronectin or fibrinogen had been adsorbed. PMN stimulated with TNF or FMLP adhered and formed zones of close apposition to fibrin, as measured by the exclusion of a 10-kD rhodamine–polyethylene glycol probe from the contact zones between PMN and the underlying fibrin gel. By this measure, IL-8– or LTB4-treated PMN adhered loosely to fibrin, since 10 kD rhodamine–polyethylene glycol permeated into the contact zones between these cells and the underlying fibrin gel. PMN stimulated with FMLP and IL-8, or FMLP and LTB4, exhibited very little migration through fibrin gels, and three times as many of these cells excluded 10 kD rhodamine–polyethylene glycol from their zones of contact with fibrin as PMN stimulated with IL-8 or LTB4 alone. These results show that PMN chemotaxis is regulated by both the nature of the chemoattractant and the composition of the extracellular matrix; they suggest that certain combinations of chemoattractants and matrix proteins may limit leukocyte movements and promote their localization in specific tissues in vivo.

Soluble or cell-bound chemoattractants (1, 2) stimulate PMN to emigrate from the vasculature and migrate toward sites of injury, infection, and inflammation. PMN express unique plasma membrane receptors for many different chemoattractants and cytokines (e.g., IL-8, leukotriene B4 [LTB4[1]], FMLP, and TNF) (3). Interactions between these receptors and soluble or surface-bound chemoattractants or cytokines signal PMN to alter their expression and/or activity of selectins and integrins (4, 5) and regulate PMN spatial orientation and movements (6).

The evolution of many chemically distinct chemoattractants and receptors suggested to us that, in addition to promoting adhesion and directing PMN locomotion, these molecules might regulate the strength of PMN adhesion to specific matrix proteins. Our findings that TNF stimulates PMN to adhere to fibrinogen-coated surfaces via CD11c/CD18 (7, 8), while phorbol dibutyrate stimulates PMN to adhere to these surfaces via CD11b/CD18 (8, 9), prompted us to examine the effects of different chemoattractants on PMN migration through three dimensional matrices composed of fibrin, collagen IV, or Matrigel (Collaborative Research, Inc., Waltham, MA), and through gels formed by thrombin treatment of cell-free plasma.

Materials and Methods

Reagents. Human monocyte IL-8 (Ser–IL-8)$_{72}$ and TNF were from Upstate Biotechnology, Inc. (Lake Placid, NY). LTB4, FMLP and Ficoll-Hypaque were from Sigma (St. Louis, MO). Rhodamine-conjugated polyethylene glycol was prepared as described (10).

Preparation of Boyden-type Chemotaxis Chambers. Cell culture inserts (pore sizes 3 or 8 μm; Becton Dickinson & Co., Mountain

[1] *Abbreviations used in this paper:* LTB4, leukotriene B4; PBS, phosphate-buffered saline supplemented with Ca^{2+} and Mg^{2+}; PBSG-HSA, PBS supplemented with 0.1% human serum albumin, and 5.5 mM glucose; Rh-PEG, rhodamine-conjugated polyethylene glycol.

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View, CA) were overlaid with the following proteins: Fibrin gels: 1 U of thrombin (a gift from Dr. John Fenton, Albany Medical College, Albany, NY) in 5 μl of PBS was added first to each insert. 0.1 ml phosphate-buffered saline supplemented with Ca^{2+} and $Mg^{2}+$ (PBS), containing 100 μg commercial grade fibrinogen (Calbiochem-Novabiochem, San Diego, CA) or purified fibrinogen (a gift from Dr. Jeffrey Weitz, MacMaster University, Hamilton, Ontario), was then placed into each 8.2-mm diameter insert on top of the thrombin. (During the course of these studies, Becton Dickinson & Co. changed the configuration of the inserts, reducing their inner diameter from 8.2 to 6.4 mm. The amount of fibrinogen added to each type of cell culture insert was maintained at ~1.9 μg fibrinogen/mm^2.) The mixture was incubated at 37°C for 5 min to allow fibrin gel formation (determined by visual inspection). 1 U D-phenyl-analyl-L-propyl-L-arginine chloromethyl ketone (PPACK) (10^{-5} M final concentration; Calbiochem-Novabiochem) in 100 μl medium was added to each insert to inhibit thrombin, and gels were washed with 250 μl PBS to remove inactivated thrombin. The fibrin gels formed were ~1 mm thick, as measured under a dissecting microscope. Collagen type IV and Matrigel matrices: 0.1 ml PBS containing 100 μg human placental collagen IV (Fluka Chemical Corp., Ronkonkoma, NY), or 80 μg of reconstituted basement membrane proteins (Matrigel), was placed into each insert and allowed to gel at room temperature for 24 h. Clotted plasma: Whole blood was collected, and the cellular components were removed by centrifugation. The resulting plasma was mixed with an equal volume of PBS, and 100 μl of this mixture was placed into each insert containing thrombin and allowed to clot as described above. One U of PPACK in 100 μl PBS then was added, and the inserts were washed with 250 μl of PBS. Fibrinogen or fibronectin: 0.1 ml of PBS containing 100 μg/ml fibrinogen or fibronectin (New York Blood Center, New York), was placed into each insert (pore size 3 μm). Inserts were incubated at 37°C for 60 min and washed with 250 μl of PBS. Filters coated with fibrinogen or fibronectin were diffusely fluorescent as visualized by epifluorescent microscopy when incubated with the corresponding antibody (fluorescein-labeled anti-fibrinogen, or anti-fibronectin mAbs; Cappel Laboratories, Malvern, PA), while uncoated filters, or filters incubated with fluorescein-labeled antibody of the opposite specificity, were not.

PMN Migration. PMN were prepared from fresh heparinized blood from healthy adult volunteers by sedimentation on Ficoll-Hypaque gradients. Contaminating RBC were removed by hypotonic lysis, as described (7). The purity of PMN isolated by this method is >95%, as determined by Wright-Giemsa staining (7). 10^6 PMN in 250 μl of PBS supplemented with 5.5 mM glucose and 0.1% human serum albumin (PBSG-HSA), were placed in the upper compartment of each insert and incubated for 0–6 h at 37°C in a humidified atmosphere containing 95% air/5% CO_2. At the times and concentrations specified, chemoattractants/cytokines were added to the top or bottom compartment in 250 μl of PBSG-HSA. At the end of the incubation, the chambers were shaken to dislodge PMN from the lower surface of the inserts. The medium in each lower compartment was collected and its content of PMN was determined using either a Coulter counter (Coulter Corp., Hialeah, FL) or a hemocytometer. Both methods gave similar results. Counts are expressed as the average number of PMN that migrated into the lower compartment. Unless otherwise indicated, all values reported are the average of six data points from at least three independent experiments.

Confocal Microscopy. PMN were suspended in medium containing 10 μM calcein/acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR), 0.02% (wt/vol) pluronic F-127 (Molecular Probes, Inc.), 2% heat-inactivated calf serum (Hyclone Laboratories, Inc., Logan, UT), and 0.2% DMSO, and mixed gently for 40 min at room temperature. Cells loaded with dye under these conditions exhibited no changes in motility (J. T. H. Mandeville and F. R. Maxfield, unpublished observations). The calcein-loaded cells were rinsed in PBSG-HSA and added to inserts containing fibrin gels in the presence or absence of TNF, FMLP, LTB4, or IL-8. After incubation with PMN, fibrin-coated filters were gently cut from their inserts using a razor blade, transferred to a glass slide, immersed in PBSG-HSA, and covered with a glass coverslip. Migration of calcein-loaded PMN through fibrin was analyzed using a microscope (Dialux 20×; E. Leitz, Inc., Rockleigh, NJ) fitted with a K2 Bio confocal scanning optical attachment using a Nipkow spinning disk. The microscope was equipped with an image intensifier, charge-coupled device camera, and video frame averager. The surface of the fibrin gel was identified using reflection interference contrast microscopy. Cells were imaged with a Plan-neofluor 25× fluorescence objective (numerical aperture = 0.8), using fluorescein optics (490-nm excitation, 525-nm emission) and a spinning disk with pinhole apertures. Serial confocal optical sections were acquired at 1-μm intervals, digitized using the VolCon program (a PC-based image processing package; Indec, Capitola, CA). Three-dimensional images were volume rendered using Microvoxel software (Indec) after passing data through a 3 × 3 × 3 Gaussian convolution filter. Each experiment was repeated at least twice using duplicate samples.

PMN Adhesion to Fibrin-coated Surfaces. Fibrin-coated Terasaki tissue culture plates were prepared as described (10). 5 μl of PBSG-HSA, containing PMN (10^6/ml) and the indicated chemoattractant, was added to each well of the plate. Plates were incubated at 4°C for 30 min to allow PMN to settle to the bottom of the wells, and were warmed to 37°C for 15 min to allow PMN to adhere. Nonadherent cells were removed as described (7), and 2.5% glutaraldehyde in PBS was added to fix the adherent PMN. PMN adherent to each well were enumerated using a phase-contrast microscope. Values reported are the mean number of PMN adherent to six wells from a representative experiment (n = 3).

Exclusion of Rhodamine-conjugated Polyethylene Glycol (Rh-PEG) from Zones of Adhesion of PMN to Protein-coated Surfaces. 10 kD Rh-PEG, prepared and used as described previously (10), does not bind to untreated glass, tissue culture plastic, or to cell membranes. Rh-PEG binds avidly to protein-coated surfaces and can be detected easily by its fluorescence. Individual wells on glass microslides (Carlson Scientific, Peotone, IL) were coated with either fibrin, Matrigel, or collagen IV, in a manner similar to that for coating cell culture inserts, except that 20 μl of the various solutions were used per well. 20 μl of PMN (10^6 cells/ml in PBSG-HSA) were added to each well, and PMN were allowed to adhere for 15 min at 37°C. The cells were washed in PBS, fixed with 3.7% paraformaldehyde in PBS for 10 min, washed again with PBS, and further incubated with 10 kD Rh-PEG at room temperature for 60 min. The preparation was then washed with PBS and immediately observed by phase and fluorescent microscopy at a magnification of 400. Average values from three different experiments are reported as the percentage of PMN that excluded Rh-PEG from zones of adherence between the cells and the underlying matrix.

Degradation of ^{125}I-labeled Fibrin Gels. 1.0 ml PBS containing 1 mg human fibrinogen, 1 mCi of $Na^{125}I$ (NEN Laboratories, Boston, MA), and 1 Iodobead (Pierce Chemical Co., Rockford, IL), was incubated for 15 min on ice. ^{125}I-fibrinogen was separated from ^{125}I by gel filtration over a Speedy Desalting Column (Pierce Chemical Co.). >97% of the ^{125}I recovered in the fibrinogen-containing fractions was precipitable with 20% TCA. 5 μl PBS

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containing 1 U of thrombin, followed by 0.1 ml PBS containing 10^6 cpm of ^{125}I-fibrinogen (~10 μg) and 100 μg unlabeled fibrinogen, were added to each insert, as described above. The resulting ^{125}I-labeled fibrin gels were treated with PPACK, washed, and incubated with 10^6 PMN, as described in the text. At various times after PMN addition, the medium was removed from the upper and lower compartments and added to 0.1 ml of PBS containing 10 mg/ml BSA. Ice-cold TCA was added to a final concentration of 20%, and samples were centrifuged to sediment acid-insoluble materials. TCA-soluble and insoluble materials were separated by centrifugation and ^{125}I in each fraction was determined using a minigamma counter (LKB Instruments, Inc., Gaithersburg, MD).

Results

IL-8 and LTB4, but Not TNF or FMLP, Promote the Migration of PMN through Fibrin Gels. PMN were placed into the upper compartment of inserts containing fibrin gels. IL-8, LTB4, FMLP, or TNF was placed in the medium in the lower compartment, and the chambers were incubated at 37°C for 6 h. IL-8 or LTB4 stimulated 12–25% of PMN to migrate through the fibrin gels and into the lower compartment. In the absence of a chemoattractant, or in response to various concentrations of TNF (10^{-9}–5×10^{-6} M) or FMLP (10^{-10}–10^{-6} M), <0.3% of the PMN migrated through fibrin gels into the lower compartments (Figs. 1 *A* and 2 *A*). Moreover, PMN did not migrate through fibrin in response to the addition of 2–10% zymosan-activated human plasma (C5a) in the lower compartment (data not shown).

PMN stimulated by IL-8 or LTB4, but not by TNF or FMLP, migrated through fibrin gels formed by thrombin treatment of commercial-grade fibrinogen (Fig. 1 *A*) or of purified fibrinogen (data not shown), or through plasma gels formed by thrombin treatment of human plasma (Fig. 1 *B*). Moreover, the presence of 20% human serum in the medium in both upper and lower compartments did not alter PMN migration through fibrin gels in response to IL-8, nor did the presence of serum promote PMN migration through fibrin gels in response to TNF or FMLP (data not shown). That PMN migrate through fibrin gels in the presence of human serum and through gels formed from whole human plasma, indicates that IL-8 promotes PMN migration through fibrin gels containing the complex mixture of plasma proteins found under physiological conditions.

The percentage of PMN that migrated through fibrin gels varied with the concentration of IL-8 or LTB4 placed in the bottom compartment (Fig. 2 *B*). Maximal PMN migration occurred with 0.7×10^{-7} M IL-8 or 0.2×10^{-7} M LTB4 (Fig. 2 *B*). PMN migration decreased dramatically when IL-8 was used at concentrations $>10^{-7}$ M, consistent with the report of Smith et al. (11) that high concentrations of IL-8 desensitize PMN. In contrast, there was no indication of PMN desensitization in response to supraoptimal concentrations of LTB4 (Fig. 2 *B*).

To determine whether IL-8 and LTB4 promote PMN migration through fibrin gels by stimulating chemotaxis or chemokinesis, we performed a checkerboard-type analysis (12). Few PMN migrated through fibrin gels when IL-8 or LTB4 was placed in the upper compartment, or when the upper and lower compartments contained equal concentrations of IL-8 or LTB4 (Fig. 1). As the difference in IL-8 or LTB4 concentrations between the upper and lower compartments decreased, the number of PMN that migrated through the fibrin gels also decreased (Fig. 3). These results indicate that PMN migration through fibrin gels in response to IL-8 or LTB4 reflects chemotaxis, not chemokinesis.

Between 25 and 50% more PMN migrated through fibrin in response to LTB4 than to IL-8. It is unlikely that this difference reflects the response of different PMN subpopulations to LTB4 vs IL-8, since the same percentage of PMN traversed fibrin gels in response to optimal concentrations of both LTB4 and IL-8 in the lower compartment as to LTB4 alone (data not shown). Other investigators have reported that only 20–50% of PMN migrate through filters (13), natural matrices, and cellular barriers (14) when stimulated by these chemoattractants. Since virtually all PMN orient and crawl on surfaces when exposed to the chemoattractants (3), it is evident that all PMN responded to them. The reason(s) why only a fraction of PMN migrate through artificial or natural barriers in response to chemoattractants is unknown.

PMN migrated through fibrin gels more rapidly in response to an optimal concentration of LTB4 than to an optimal con-

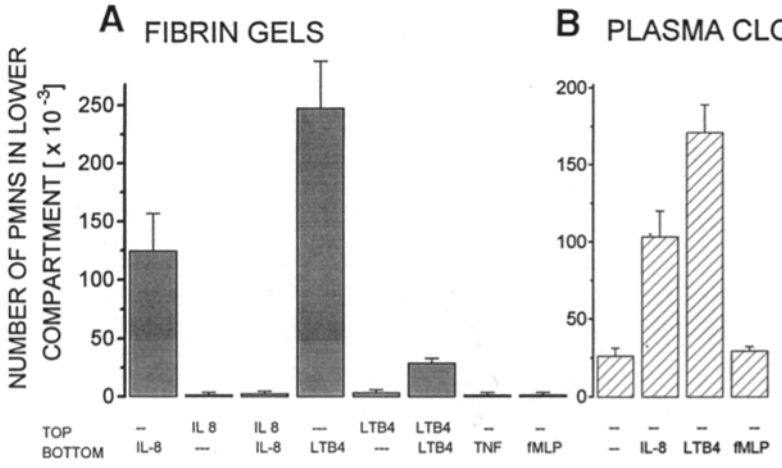

Figure 1. IL-8 and LTB4 promote PMN migration through fibrin gels and plasma clots. Fibrin gels (*A*) or plasma clots (*B*) were formed on top of filters with 8-μm pores in tissue culture inserts, as described in Materials and Methods. 10^6 PMN were added to the upper chamber, and the indicated chemoattractants or cytokines were added to either the lower or upper chamber as indicated. The preparation then was incubated at 37°C for 6 h, at which time the number of cells in the lower chamber was determined using a Coulter counter. Concentrations of chemoattractants/cytokines used were: 0.75×10^{-7} M (IL-8), 1.0×10^{-7} M (LTB4), 0.5×10^{-7} M (TNF), and 1.0×10^{-7} M (FMLP). Fewer than 1,500 PMN migrated through fibrin gels in the absence of any stimulator.

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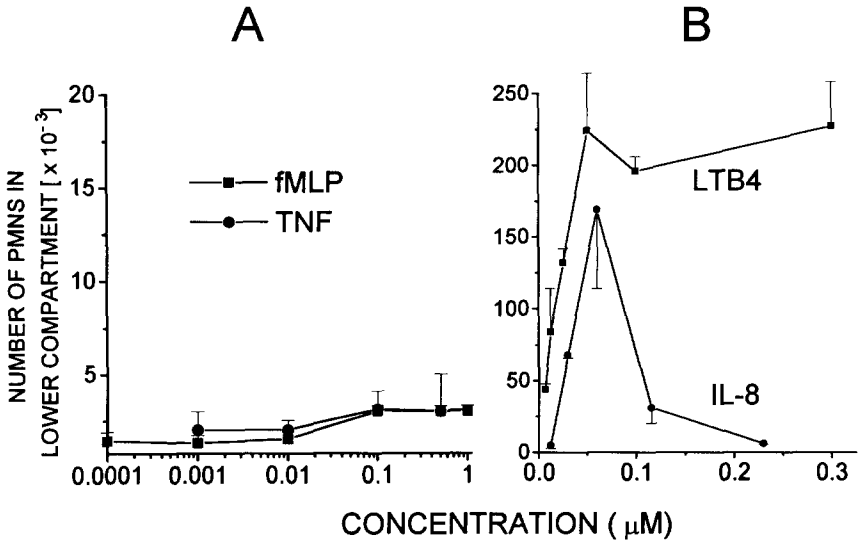

Figure 2. PMN migration through fibrin gels in response to varying concentrations of chemoattractants. Fibrin gels were formed on filters of cell culture inserts, as described in Materials and Methods, and chemoattractants or cytokines at the indicated concentrations were added to the lower chamber. 10^6 PMN were added to the upper chamber, and the preparation was incubated at 37°C for 6 h, at which time the number of cells in the lower chamber was counted as described in Fig. 1.

centration of IL-8 (Fig. 4). 10% of LTB4-stimulated PMN migrated through fibrin gels within 2 h, while <0.5% of IL-8–stimulated PMN migrated through these gels in this time period (Fig. 4). By 6 h, maximal numbers of PMN had migrated through fibrin gels in response to either IL-8 or LTB4.

To visualize the interactions of chemoattractant-stimulated PMN with fibrin gels, PMN prelabeled with calcein (15) were added to the upper compartment of inserts containing fibrin gels. Chemoattractants were added to the medium in the lower compartment, the chambers were incubated at 37°C, and at the times indicated the fibrin-coated filters were removed and examined by confocal microscopy. After a 1- or 4-h incubation with FMLP or TNF, almost all the cells remained on the gel's surface; <5% of TNF- or FMLP-stimulated PMN penetrated a short distance into the fibrin gels (Fig. 5). In contrast, >80% of IL-8–stimulated PMN migrated deeply into the fibrin gels after a 4-h incubation (Fig. 5). Greater than 80% of LTB4-stimulated PMN began to migrate into the fibrin gel after 1 h, while few IL-8–stimulated PMN penetrated the fibrin at this time (data not shown). These results show that TNF and FMLP do not promote PMN invasion of fibrin, and that LTB4 stimulates PMN to enter fibrin gels more rapidly than IL-8. The latter finding is consistent with the more rapid transit of fibrin gels by LTB4- than IL-8–stimulated PMN described in Fig. 4.

To further examine whether proteolysis of fibrin accounted for the selective ability of IL-8– or LTB4-stimulated PMN to traverse these gels, we measured the release of ^{125}I-labeled products from ^{125}I-fibrin incubated with PMN for 6 h at 37°C in the presence or absence of each of these chemoattractants. The rate and extent of release of ^{125}I-labeled acid-soluble and acid-precipitable products were similar for all four chemoattractants (Fig. 6). Even in the presence of 20% serum, chemoattractant-stimulated PMN released no more ^{125}I-

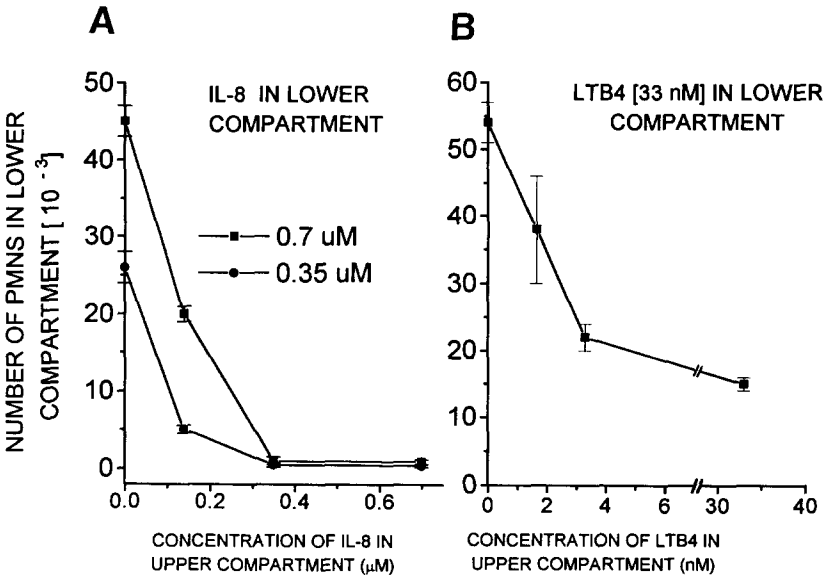

Figure 3. An IL-8 or LTB4 gradient is required for PMN migration through fibrin gels. Fibrin gels were formed in cell culture inserts, as described in Materials and Methods. IL-8 (*A*) or LTB4 (*B*) were added to the lower compartment at a fixed concentration and to the upper compartment at varying concentrations as indicated. 10^6 PMN were added to the upper chamber, and the preparation was incubated at 37°C for 6 h, at which time the number of cells in the lower chamber was counted as described in Fig. 1.

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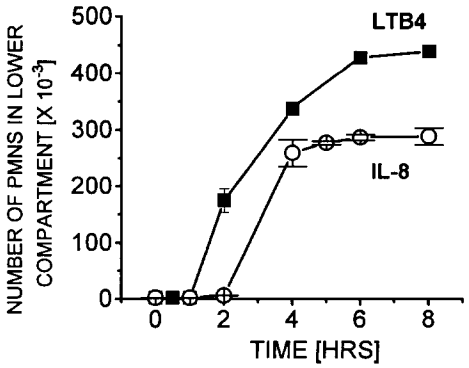

Figure 4. Time course of PMN migration through fibrin gels. Fibrin gels were formed in cell culture inserts, as described in Materials and Methods. IL-8 (0.75×10^{-7} M) or LTB4 (10^{-7} M) was added to the lower chamber. The number of PMN that migrated through a fibrin gel was determined at the indicated times, as described in Fig. 1.

labeled products than did unstimulated PMN. These results suggest that fibrin degradation does not account for the selective ability of LTB4- and IL-8–stimulated PMN to traverse fibrin gels.

LTB4, IL-8 TNF, and FMLP Promote PMN Migration through Gels Formed of Matrigel and Collagen IV. To confirm that the inability of TNF-, FMLP-, or zymosan-activated human plasma–stimulated PMN to migrate through fibrin gels reflected an effect of the interaction between the fibrin matrix and chemoattractant-stimulated PMN, and not a general effect of any three-dimensional matrix on PMN stimulated with these chemoattractants, we examined whether TNF, zymosan-activated human plasma, and FMLP promoted PMN migration through gels composed of basement membrane proteins (Matrigel) or collagen IV (Fig. 7). TNF, FMLP, IL-8, zymosan-activated human plasma, or LTB4, added to the bottom chamber, stimulated PMN migration through these gels (Fig. 7 *A* and data not shown). To determine whether fibrin affected PMN migration through collagen matrices, inserts coated with collagen IV gels were incubated with fibrinogen and thrombin to form a fibrin layer on top of the collagen gels, and washed with PPACK-containing buffer. PMN were added to the upper compartment and TNF to the lower compartment. The presence of fibrin prevented PMN migration through the collagen gels in response to TNF by ~75% (Fig. 7 *A*). These results confirm that the effect of fibrin is selective and that it affects PMN migration in response to a specific subset of chemoattractants.

To determine whether protein monolayers had the same effects on PMN migration as gels, inserts were coated with fibrinogen or fibronectin. The adsorption of these proteins to the filters that form the floor of the inserts was confirmed by immunofluorescence microscopy, as described in Materials and Methods. FMLP, TNF, IL-8, and LTB4 all promoted PMN migration through filters to which fibrinogen or fibronectin had been adsorbed (Fig. 7 *B*).

PMN Adhere More Closely to Fibrin in response to FMLP or TNF than to LTB4 or IL-8. Is there a relationship between the ability of a chemoattractant to stimulate PMN migration through fibrin and its ability to promote close apposition of PMN to fibrin? PMN were incubated on fibrin-coated surfaces in the presence or absence of a chemoattractant for 15 min at 37°C. As expected, TNF, FMLP, LTB4, and IL-8 were equally effective in stimulating PMN adherence to fibrin (>200 chemoattractant-stimulated PMN vs ~10 unstimulated PMN/mm^2 adhered). The closeness of PMN adhesion to fibrin was evaluated by the ability of 10 kD Rh-PEG (8, 10) to penetrate into the zones of adhesion between chemoattractant-stimulated PMN and fibrin. By this measure 70–80% of adherent TNF- or FMLP-stimulated PMN excluded Rh-PEG from their zones of contact with the fibrin (Fig. 8). In contrast, only ~15% of adherent LTB4- or IL-8–stimulated PMN formed adhesive zones that excluded Rh-PEG (Fig. 8). Furthermore, the adhesive zones formed by this 15% of IL-8–stimulated PMN were at least 50% smaller in area than those formed by TNF- or FMLP-stimulated PMN, as judged by the area from which Rh-PEG was excluded (data not shown).

Our previous work (10) showed that the exclusion of fluorescein-conjugated F(ab)$_2$ anti-fibrinogen from zones of contact between ADP-stimulated platelets and fibrinogen-coated surfaces is a useful measure of the closeness of apposition between platelet membranes and the substrate. Therefore, we used the exclusion of fluorescein-conjugated F(ab)$_2$ anti-fibrin from zones of contact between LTB4- or IL-8–stimulated PMN and fibrin as a measure of the interaction of these cells with fibrin-coated surfaces. About 50% of fibrin-adherent LTB4- or IL-8–stimulated PMN formed adhesive zones that excluded this high molecular weight (100-kD) probe (data not shown). As expected from studies with Rh-PEG (Fig. 8), >99% of fibrin-adherent TNF- or FMLP-stimulated PMN excluded fluorescein-conjugated F(ab)$_2$ anti-fibrin from their zones of contact with fibrin (data not shown). Thus, LTB4- or IL-8–stimulated PMN adhere more closely to fibrin than do unstimulated PMN, even though these chemoattractants do not promote the very close apposition characteristic of FMLP- or TNF–stimulated PMN.

The Effect of Combinations of Chemoattractants on Migration of PMN through Fibrin Gels. The inability of FMLP- or TNF–stimulated PMN to migrate through fibrin can be interpreted in at least two ways. First, fibrin blocks the capacity of PMN to respond to FMLP or TNF. This seems unlikely, since FMLP and TNF promote close apposition between PMN and fibrin-coated surfaces (Fig. 8). Second, FMLP or TNF signal PMN to become sessile when they interact with fibrin. To examine the second possibility, we monitored the effects of combinations of chemoattractants on PMN migration through fibrin gels (Fig. 9). The presence of FMLP in the bottom compartment of the inserts reduced PMN migration through fibrin gels in response to IL-8 or LTB4 in a concentration-dependent fashion. Higher concentrations of FMLP were required to effect equal inhibition of migration of LTB4-stimulated PMN vs IL-8–stimulated PMN (Fig. 9). TNF had a small, reproducible, but statistically insignificant inhibitory effect on the migration of PMN in response to IL-8 and no measurable effect on PMN migration in response to LTB4.

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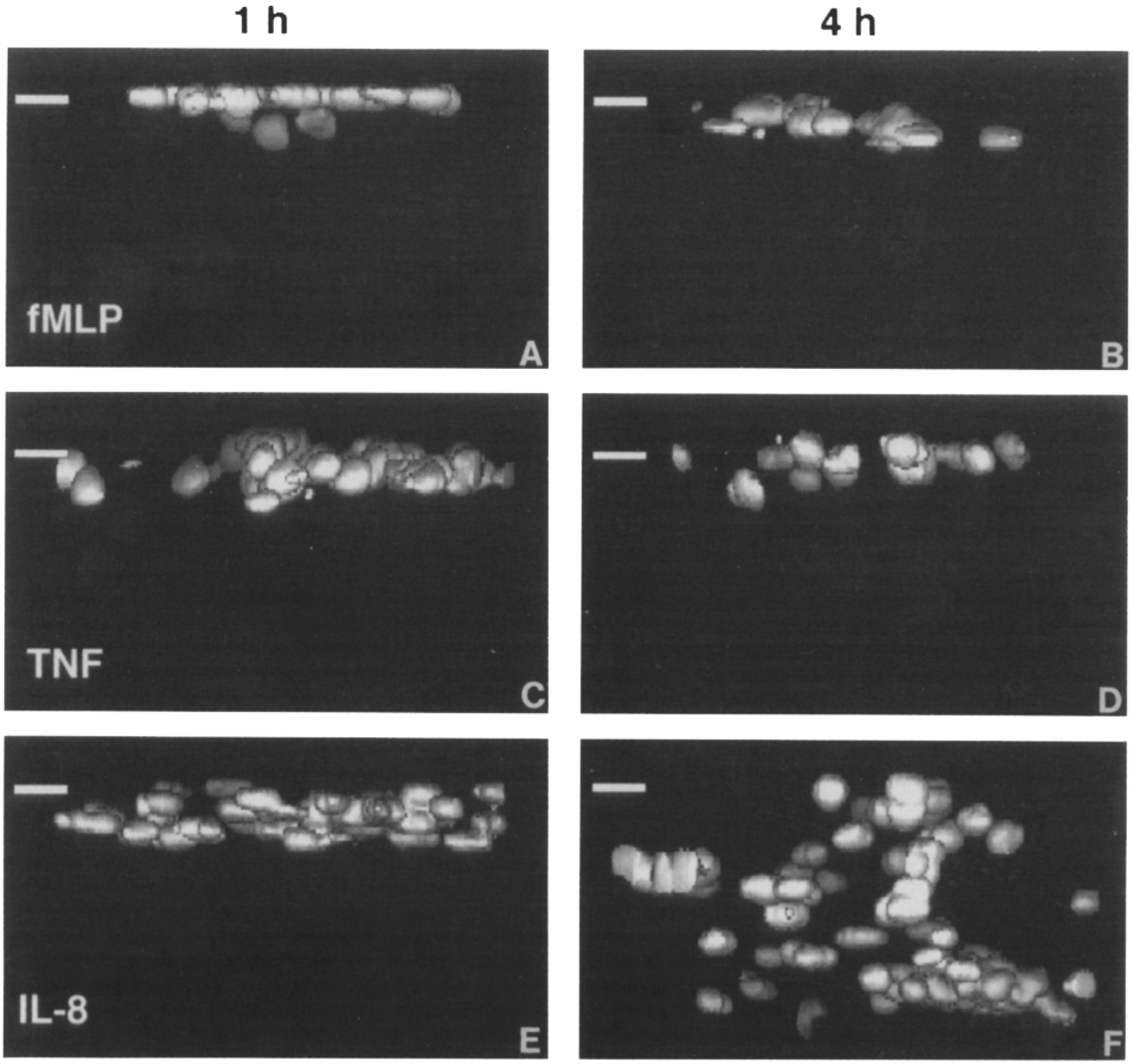

Figure 5. Confocal microscopic analysis of PMN migration through fibrin gels. Fibrin gels were formed as described in Materials and Methods. At the indicated concentrations, chemoattractants or cytokines were added to the lower chamber. 10^6 PMN, prelabeled with calcein as described in Materials and Methods, were added to the upper chamber at 37°C. At the indicated times, the filters were removed from the inserts, washed, and viewed by confocal microscopy, as described in Materials and Methods. All samples were viewed en face; the images were rotated 90°. The surface of the gel is marked with a bar. *A* and *B*, FMLP (10^{-7} M); *C* and *D*, TNF (0.5×10^{-7} M); *E* and *F*, IL-8 (0.75×10^{-7} M).

Thus, FMLP selectively reduced PMN migration through fibrin gels in response to IL-8 or LTB4 (Fig. 9).

We also examined the effects of combinations of chemoattractants on close apposition of PMN with fibrin. FMLP, in combination with IL-8 or LTB4, induced ~50% of PMN to form zones of adhesion that excluded 10-kD Rh-PEG (Fig. 8). In contrast, only 15% of PMN stimulated with IL-8 or LTB4 alone formed closely apposed zones of adhesion (Fig. 8). Thus, the capacity of PMN to form close zones of adhesion on fibrin was inversely associated with the capacity of PMN to migrate through fibrin gels under conditions where PMN were stimulated with TNF, FMLP, IL-8, or LTB4 alone or with FMLP in combination with IL-8 or LTB4.

Discussion

Matrix Proteins Modulate Cellular Responses to Hormones, Cytokines, and Growth Factors. Matrix proteins exert profound effects on adhesion, differentiation, migration, and/or secre-

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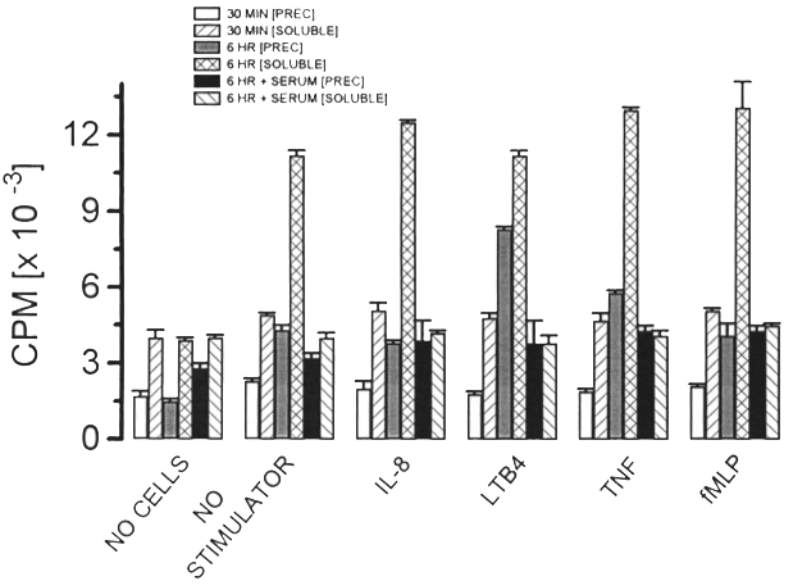

Figure 6. Degradation of ^{125}I-fibrin by PMN. Cell culture inserts containing ^{125}I-labeled fibrin gels were prepared as described. 10^6 PMN were added to the insert, and the indicated chemoattractants/cytokine were added to the lower compartment as described in Fig. 1. The concentrations of chemoattractants used are the same as described in Fig. 5. After a 6-h incubation at 37°, the media from both the upper and lower compartments were collected and assayed for the amount of radioactivity in TCA-soluble and insoluble fractions, as described in Materials and Methods. This figure is representative of experiments repeated three times with similar results. The data in this figure are the average of values from duplicate samples.

tion of epithelial cells (16, 17), endothelial cells (18), neurons (19, 20), and leukocytes (7, 9, 21–27). Matrix proteins also affect the ability of many types of cells to respond to hormones, growth factors, and cytokines (28, 29). Our findings that some chemoattractants (e.g., FMLP, TNF, C5a), promote PMN migration in the context of two types of extracellular matrix proteins (e.g., matrigel and collagens IV) (Fig. 7 and data not shown), and PMN immobilization, in the context of another (e.g., fibrin) (Figs. 1 *A*, and *B*, 2 *A*, and 5 and data not shown), are the first to show that specific matrix proteins regulate leukocyte chemotaxis. These results show that fibrin gels, fibrin-impregnated collagen gels, and fibrin-containing plasma clots present selective barriers to the migration of FMLP-or TNF-stimulated PMN, and that chemotaxis of PMN through three-dimensional matrices is regulated by both the specific chemoattractant and the protein composition of the matrix with which the cells are in contact.

Matrix Proteins Regulate PMN Adhesion, Phagocytosis, and Secretion. We (7, 9) and others (21) have shown that TNF or phorbol ester–stimulated PMN adhere to fibrinogen-coated surfaces via different β2 integrins (CDllb/CD18 vs CDllc/CD18, respectively), and Lundgren-Akerlund et al. (22) and Thompson and Matsushima (23), have reported that FMLP-stimulated PMN adhere to protein-coated surfaces with different efficiencies depending on the matrix protein used to coat these surfaces. With respect to phagocytosis, Pommier et al.

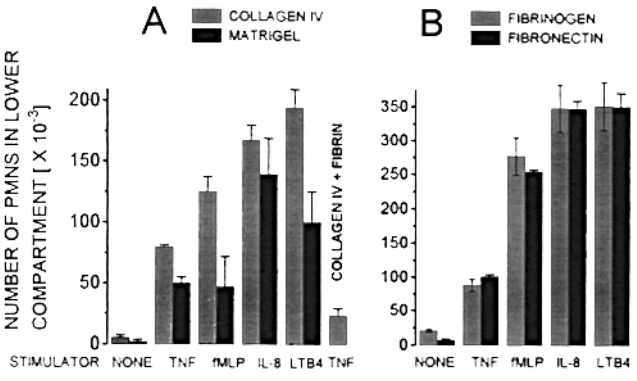

Figure 7. PMN migration through filters coated with different types of matrix proteins. PMN migration through filters coated with gels formed with reconstituted basement membrane (Matrigel), or collagen IV (*A*), or with filters coated with fibrinogen or fibronectin (*B*) was measured in response to TNF (5×10^{-7} M), FMLP (10^{-7} M), IL-8 (0.75×10^{-7} M), or LTB4 (10^{-7} M). Migration was essentially complete within 2 h. The last column of Fig. 5 *A* reports migration of TNF-stimulated PMN through a collagen IV gel impregnated with fibrin.

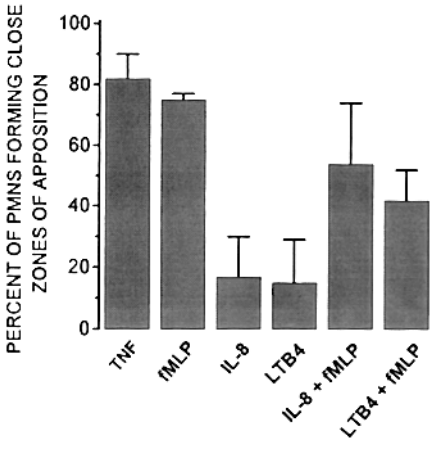

Figure 8. "Closeness" of PMN apposition to fibrin matrices. PMN stimulated with the indicated chemoattractants were allowed to adhere for 15 min to glass surfaces coated with fibrin, as described in Materials and Methods. PMN forming close zones of adhesion are defined as those that exclude the entry of Rh-PEG into the area of adhesion between the cell and the underlying matrix as assayed by fluorescence microscopy. The concentrations of chemoattractants/cytokines used were the same as in Fig. 5. Less than 20% of unstimulated PMN that adhered to the fibrin formed close zones of apposition.

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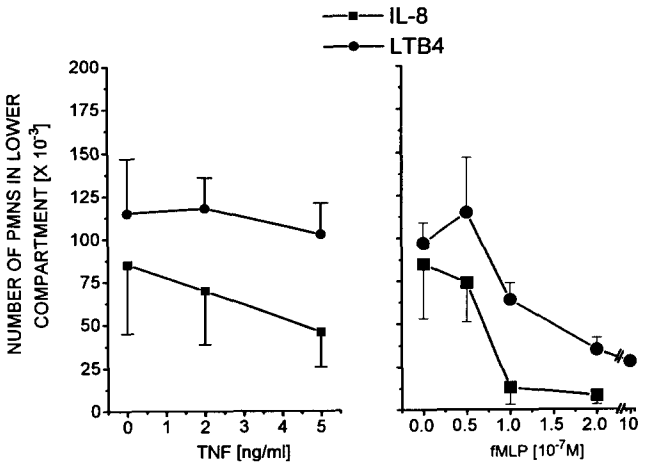

Figure 9. The effect of combinations of chemoattractants/cytokines in promoting PMN migration through fibrin gels. PMN migration through fibrin gels was measured in response to chemoattractants added in combination to the bottom compartment at the concentrations indicated. The concentrations of IL-8 and LTB4 used were the same as in Fig. 5. PMN migration into the lower compartment was measured as described in Fig. 1 after a 6-h incubation at 37°C.

(24) and Wright et al. (25) showed that the interaction of fibronectin with its $\beta 1$ integrin activates complement receptors (CD11b/CD18) on monocytes and PMN to phagocytose C3bi-coated particles. With respect to secretion, Monboisse et al., (26, 27) reported that the interaction of unstimulated or chemoattractant-stimulated PMN with collagen I-coated surfaces induces the secretion of proteolytic enzymes and O_2^-. In contrast, preincubation of PMN with collagen IV blocks the ability of collagen I and FMLP to stimulate resting PMN to secrete these products (26, 27). Similarly, adhesion of TNF-stimulated PMN to extracellular matrix proteins that express Arg-Gly-Asp motifs enhances PMN secretion (30). The findings reported here add chemotaxis to the list of leukocyte functions modulated by their contact with matrix proteins.

Relationship between Strength of Adhesion, Closeness of PMN Apposition to the Substrate, and PMN Migration. DiMilla et al. (31) have explored the relationship between strength of cell adhesion to a substrate and cell migration by following the spontaneous migration of human smooth muscle cells on surfaces that had absorbed varying concentrations of fibronectin or collagen IV. Under the conditions of their experiments, the rate of cell migration was maximal at an intermediate level of cell–substratum adhesiveness. Goodman et al. (32) found a similar biphasic relationship between the movement of murine skeletal myoblasts and the absorbed concentration of laminin on the substrate.

While we have not directly measured the strength of PMN adhesion to fibrin, we have examined the "closeness" of apposition between PMNs' matrix-adherent surfaces and matrices containing different proteins by measuring the permeability of zones of contact between PMN and the underlying matrix to macromolecular probes. We defined "close" apposition as the exclusion of 10 kD Rh-PEG from zones of contact between the PMNs' substrate-adherent membranes and the matrix, and "loose" apposition as permeation of 10 kD Rh-PEG into these zones. Our studies showed that chemoattractants, such as IL-8 and LTB4, elicit "loose" apposition between PMN and fibrin gels and promote PMN migration through these gels. Chemoattractants, such as FMLP and TNF, that signal "close" apposition between PMN and fibrin gels do not promote PMN migration through these gels. This correlation was further supported by our findings that PMN stimulated by any of these chemoattractants formed loose zones of adhesion (e.g., permeable to 10 kD Rh-PEG) on collagen IV or Matrigel (data not shown) and migrated through these matrices (Fig. 7 *A*), and that FMLP induced LTB4- or IL-8–stimulated PMN to form close zones of apposition to fibrin and cease migration (Figs. 8 and 9). Thus, there is an inverse association between close PMN interaction with a matrix protein and the ability of PMN to migrate though gels containing it. These findings suggest that "close" and "loose" apposition between PMN and matrix proteins, as defined here, are functionally equivalent to very strong and intermediate adhesion between cells and matrix, respectively, as defined by DiMilla et al. (31).

Fibrin Degradation Is Not Required for PMN Chemotaxis. The zones of close apposition formed between FMLP- or TNF-stimulated PMN and fibrin gels are impermeant to molecules of >10 kD, thereby excluding virtually all plasma protease inhibitors, such as $alpha_1$ antiplasmin and $alpha_2$ macroglobulin. Therefore, leukocyte proteases secreted into these zones function virtually uninhibited (33). In contrast, IL-8– or LTB4-stimulated PMN adhere more loosely to fibrin gels. Under the latter conditions, plasma protease inhibitors should have ready access to zones of contact with the substrate and inhibit the action of leukocyte proteases. Thus, FMLP- or TNF-stimulated PMN might be expected to digest fibrin gels more efficiently than IL-8– or LTB4-stimulated PMN. This was not observed (Fig. 6). We found no significant differences in the amount of radiolabel released from ^{125}I-labeled fibrin by migrating LTB4- or IL-8–stimulated PMN vs sessile FMLP- or TNF-stimulated PMN, even in the presence of 20% serum. These findings suggest that PMN migrating through fibrin gels in response to IL-8 and LTB4 do so by mechanisms other than proteolyzing these gels. Lanir et al. (34) came to a similar conclusion in their studies of guinea pig macrophage migration through fibrin gels.

That FMLP and TNF promote PMN migration through fibrinogen-coated filters (Fig. 7) is probably related to the observations that FMLP-stimulated PMN efficiently degrade substrate-adherent proteins, including fibrinogen (33) and fibronectin (35), thereby removing these proteins from the substrate and facilitating PMN movement.

How Do Matrix Proteins Regulate Leukocyte Chemotaxis? We interpret our findings as follows: Different chemoattractants activate different subsets of PMN integrins to bind to ligands on matrix proteins (7–9). The interaction of each type of activated PMN integrin, with its cognate ligand on a matrix protein, specifies a distinct set of cellular migratory or sessile responses. These responses may result from direct interaction of a matrix protein with the activated integrin or by

signals sent by the activated integrin to other integrins on the same cell. There are several instances where ligation of one type of integrin by matrix proteins modulates the activity of another type of integrin. As noted above, Pommier et al. (24) and Wright et al. (25) showed that ligation of $\beta 1$ integrins by fibronectin activates the $\beta 2$ integrin CD11b/CD18 (CR 3) on monocytes and PMN to phagocytose C3bi-coated particles. We have shown that ligation of $\alpha 5\beta 1$ on platelets by fibronectin stimulates platelets to form close zones of apposition with fibrinogen (10). Hutalia et al. (36) reported that ligation of $\alpha 5\beta 1$ integrin by Arg-Gly-Asp peptides induces the expression of matrix metalloproteinases by fibroblasts, whereas ligation of $\alpha 4\beta 1$ integrin by intact fibronectin suppresses matrix metalloproteinase expression.

In vivo inflammatory stimuli elicit the generation of multiple chemoattractants/cytokines. Our results show that a hierarchy of cellular responses is generated when different combinations of chemoattractant receptors are stimulated simultaneously. Signals generated by FMLP receptors appear to override signals produced by LTB4 or IL-8 receptors, thereby blocking the ability of LTB4 or IL-8 to stimulate PMN migration through fibrin gels (Fig. 9). In contrast, signals generated by TNF receptors have no effect on LTB4-stimulated PMN migration through fibrin gels and a very weak inhibitory effect on IL-8–stimulated PMN migration through these gels (Fig. 9).

We conclude that PMN chemotaxis through three-dimensional lattices composed of extracellular matrix proteins is regulated both by signals initiated by a specific chemoattractant, and by signals generated when specific PMN receptors interact with their cognate ligands on extracellular matrix proteins. Viewed from this perspective, each of the many different chemoattractants provides PMN both with general instructions to crawl, and with specific instructions to become sessile when specific receptors on these cells contact their cognate ligands on matrix proteins. Thus, chemoattractants provide tissue localization instructions for PMN. It seems likely that chemoattractants also provide such instructions to other types of leukocytes as well.

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References

1. Rot, A. 1991. The role of leukocyte chemotaxis in inflammation. *In* Biochemistry of Inflammation. S.W. Evans and J.T. Whicher, editors. Kluwer Academic Publishers, Dordrecht, Netherlands. 39–54.
2. Miller, M.D., and M.S. Krangel. 1992. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. *Crit. Rev. Immunol.* 12:17–46.
3. Snyderman, R., and R.J. Uhing. 1992. Chemoattractant stimulus-response coupling. *In* Inflammation: Basic Principles and Clinical Correlates. J.I. Gallin, I.M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 421–439.
4. Kishimoto, T.K., and D.C. Anderson. 1992. The role of integrins in inflammation. *In* Inflammation: Basic Principles and Clinical Correlates. J.I. Gallin, I.M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 353–406.
5. Lasky, L.A., and S.D. Rosen. 1992. The selectins. Carbohydrate binding adhesion molecules of the immune system. *In* Inflammation: Basic Principles and Clinical Correlates. J.I. Gallin, I.M. Goldstein, and R. Snyderman, editors. Raven Press, New York. 407–420.
6. Downey, G.P. 1994. Mechanisms of leukocyte motility and chemotaxis. *Curr. Opin. Immunol.* 6:113–124.
7. Loike, J.D., B. Sodeik, L. Cao, S. Leucona, J.I. Weitz, P.A. Detmers, S.D. Wright, and S.C. Silverstein. 1991. CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the Aα chain of fibrinogen. *Proc. Natl. Acad. Sci. USA.* 88:1044–1048.
8. Loike, J.D., R. Silverstein, S.D. Wright, J.I. Weitz, and S.C. Silverstein. 1992. The role of protected extracellular compartments in interactions between leukocytes, and platelets and fibrin/fibrinogen matrices. *Ann. NY Acad Sci.* 667:163–172.
9. Wright, S.D., J.I. Weitz, A.J. Huang, J.M. Levin, S.C. Silverstein, and J.D. Loike. 1988. Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. *Proc. Natl. Acad. Sci. USA.* 85:7734–7738.
10. Loike, J.D., R. Silverstein, L. Cao, L. Solomon, J. Weitz, E. Haber, G.R. Matsueda, M.S. Bernatowicz, and S.C. Silverstein. 1993. Activated platelets form protected zones of adhesion with fibrinogen and fibronectin-coated surfaces. *J. Cell Biol.* 121: 945–955.
11. Smith, W.B., J.R. Gamble, I. Clark-Lewis, and M.A. Vadas. 1993. Chemotactic desensitization of neutrophils demonstrates IL-8 dependent and IL-8 independent mechanisms of transmigration through cytokine activated endothelium. *Immunology.*

78:491–497.
12. Zigmond, S.H., and J.G. Hirsch. 1973. Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. *J. Exp. Med.* 137: 387–410.
13. Harvath, L., and E.J. Leonard. 1982. Two neutrophil populations in human blood with different chemotactic activities: separation and chemoattractant binding. *Infect. Immun.* 36:443– 449.
14. Furie, M.B., B.L. Naperstek, and S.C. Silverstein. 1987. Migration of neutrophils across monolayers of cultured microvascular endothelial cells. An in vitro model of leucocyte extravasation. *J. Cell Sci.* 88:161–175.
15. Weston, S.A., and C.R. Parish. 1990. New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. *J. Immunol. Methods.* 133:87–97.
16. Howlett, A.R., and M.J. Bissell. 1993. The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary eptithelium. *Epithelial Cell Biol.* 2:79–89.
17. Klemke, R.L., M. Yebra, E.M. Bayna, and D.A. Cheresh. 1994. Receptor tyrosine kinase signaling required for integrin $\alpha v\beta 5$-directed cell motility but not adhesion on vitronectin. *J. Cell Biol.* 127:859–866.
18. Milici, A.J., M.B. Furie, and W.W. Carly. 1985. The formation of fenestration and channels by capillary endothelium in vitro. *Proc. Natl. Acad. Sci. USA.* 82:6181–6185.
19. Calof, A.L., and A.D. Lander. 1991. Relationship between neuronal migration and cell-substratum adhesion: laminin and merosin promote olfactory neuronal migration but are anti-adhesive. *J. Cell Biol.* 115:779–794.
20. Bronner-Fraser, M. 1994. Neural crest cell formation and migration in the developing embryo. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 8:699–706.
21. Detmers, P.A., S.K. Lo, E. Olsen-Egbert, A. Walz, M. Baggiolini, and Z.A. Cohn. 1990. Neutrophil-activating protein 1/interleukin 8 stimulates the binding activity of the leukocyte adhesion receptor CD11b/CD18 on human neutrophils. *J. Exp. Med.* 171:1155–1162.
22. Lundgren-Akerlund, E., E. Berger, and K.E. Arfors. 1992. Effect of divalent cations on adhesion of PMN to matrix molecules in vitro. *J. Leukocyte Biol.* 51:603–608.
23. Thompson, H.L., and K. Matsushima. 1992. Human polymorphonuclear leucocytes stimulated by TNF-α show increased adherence to extracellular matrix proteins which is mediated via the CD11b/18 complex. *Clin. Exp. Immunol.* 90:280–285.
24. Pommier, C.G., S. Inada, L.F. Fries, T. Takahashi, M.M. Frank, and E.J. Brown. 1983. Plasma fibronectin enhances phagocytosis of opsonized particles by human peripheral blood monocytes. *J. Exp. Med.* 157:1844–1854.
25. Wright, S.D., M.R. Licht, L.S. Craigmyle, and S.C. Silverstein. 1984. Communication between receptors for different ligands on a single cell: ligation of fibronectin receptors induces a reversible alteration in the function of complement receptors on cultured human monocytes. *J. Cell Biol.* 99: 336–339.
26. Monboisse, J.C., G. Bellon, A. Randoux, J. Duffer, and J.P. Borel. 1990. Activation of human neutrophils by type I collagen. Requirement of two different sequences. *Biochem. J.* 270:459–462.
27. Monboisse, J.-C., R. Garnotel, G. Bellon, N. Ohno, C. Perreau, J.P. Borel, and N.A. Kefalides. 1994. The $\alpha 3$ chain of type IV collagen prevents activation of human polymorphonuclear leukocytes. *J. Biol. Chem.* 269:25475–25482.
28. Nicosia, R.F., and G.P. Tuszynski. 1994. Matrix-bound thrombospondin promotes angiogenesis in vitro. *J. Cell Biol.* 124: 183–193.
29. Ohno, K., and P. Maier. 1994. Cultured rat hepatocytes adapt their cellular glycolytic activity and adenylate energy status to tissue oxygen tension: influences of extracellular matrix components, insulin, and glucagon. *J. Cell. Physiol.* 160:358–366.
30. Fuortes, M., W.-W. Jen, and C. Nathan. 1993. Adhesion-dependent protein tyrosine phosphorylation in neutrophils treated with tumor necrosis factor. *J. Cell Biol.* 120:777–784.
31. DiMilla, P.A., J.A. Stone, J.A. Quinn, S.M. Albelda, and D.A. Lauffenburger. 1993. Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J. Cell Biol.* 122:729–737.
32. Goodman, S.L., G. Risse, and K. von der Mark. 1989. The E8 subfragment of laminin promotes locomotion of myoblasts over extracellular matrix. *J. Cell Biol.* 109:799–809.
33. Weitz, J.I., A.J. Huang, S.L. Landman, S.C. Nicholson, and S.C. Silverstein. 1987. Elastase-mediated fibrinogenolysis by chemoattractant-stimulated neutrophils occurs in the presence of physiologic concentrations of antiproteinases. *J. Exp. Med.* 166:1836–1850.
34. Lanir, N., P.S. Ciano, L. Van De Water, J. McDonagh, A.N. Dvorak, and H.P. Dvorak. 1988. Macrophage migration in fibrin gel matrices. II. Effects of clotting factor XIII, fibronectin, and glycosaminoglycan content on cell migration. *J. Immunol.* 140:2340–2349.
35. Campbell, E.J., R.M. Senior, J.A. McDonald, and D.L. Cox. 1982. Proteolysis by neutrophils. Relative importance of cell-substrate contact and oxidative inactivation of proteinase inhibitors in vitro. *J. Clin. Invest.* 70:845–852.
36. Huhtala, P., M. Humpries, J. McCarthy, Z. Werb, and C. Damsky. 1994. The RGD and CS-1 containing cell binding regions of fibronectin signal opposing effects on metalloproteinase expression via $\alpha 5\beta 1$. *Mol. Biol. Cell.* 5:64*a*. (Abstr.)