Differential Regulation of β_1 Integrins by Chemoattractants Regulates Neutrophil Migration through Fibrin

John D. Loike,* Long Cao,* Sadna Budhu,* Eugene E. Marcantonio,‡ Joseph El Khoury,§ Stanley Hoffman,‖ Ted A. Yednock,¶ and Samuel C. Silverstein*

*Department of Physiology and Cellular Biophysics and ‡Department of Pathology, Columbia University College of Physicians and Surgeons, New York 10032; ‖Department of Medicine, Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29425-2229; §Department of Medicine, Beth Israel Hospital, New York 10004; and ¶Athena Neurosciences, South San Francisco, California 94080

Abstract. Chemoattractants differ in their capacity to stimulate neutrophils to adhere to and to migrate through matrices containing fibrin. Formyl methionyl leucyl phenylalanine (fMLP) stimulates neutrophils to adhere closely to, but not to migrate into, fibrin gels. Leukotriene B4 (LTB4) stimulates neutrophils to adhere loosely to and to migrate through fibrin gels. We report that $\alpha_5\beta_1$ integrins regulate the different migratory behaviors on fibrin gels of neutrophils in response to these chemoattractants. fMLP, but not LTB4, activated neutrophil β_1 integrins, as measured by binding of mAb 15/7 to an activation epitope on the β_1 integrins. Antibodies or peptides that block $\alpha_5\beta_1$ integrins prevented fMLP-stimulated neutrophils from forming zones of close apposition on fibrin and reversed fMLP's inhibitory effect on neutrophil chemotaxis through fibrin. In contrast, neither peptides nor antibodies that block β_1 integrins affected the capacity of LTB4-stimulated neutrophils to form zones of loose apposition or to migrate through fibrin gels. These results suggest that chemoattractants generate at least two different messages that direct neutrophils, and perhaps other leukocytes, to accumulate at specific anatomic sites: a general message that induces neutrophils to crawl and a specific message that prepares neutrophils to stop when they contact appropriate matrix proteins for activated β_1 integrins.

Key words: chemotaxis • neutrophils • integrins • fibrin • chemoattractants

LEUKOCYTE chemotaxis is regulated by the interactions of soluble or surface-bound chemoattractants/chemokines with cognate receptors on the leukocytes. These interactions generate intracellular signals that activate one or more of the leukocyte's adhesion-promoting receptors, thereby enabling these cells to adhere to or migrate through endothelia, epithelia, and extracellular matrices.

Neutrophils (polymorphonuclear leukocytes, PMN)[1] express a number of different adhesion-promoting surface receptors, including β_1 and β_2 integrins. β_2 integrins assume an "activated" conformation when chemoattractants, chemokines, cytokines, or growth factors bind to specific receptors for these substances on PMN (Diamond and Springer, 1994; Premack and Schall, 1996). Activation increases the capacity of β_2 integrins to bind cognate ligands on cells or matrix proteins, thereby regulating PMN adhesion to and migration through endothelia (Smith, 1993; Springer, 1995), epithelia (McCormick et al., 1995), layers of synovial fibroblasts (Gao et al., 1995; Gao and Issekutz, 1996), and extracellular matrices (Wright et al., 1988; Loike et al., 1991, 1992, 1995). The central roles played by β_2 integrins in PMN adhesion and chemotaxis in vivo are illustrated by the multiple derangements of PMN function in humans with the inherited disorder leukocyte adhesion deficiency type 1, in which there is partial to complete absence of β_2 chains (Anderson and Springer, 1987), and in mice rendered functionally or genetically deficient in $\alpha_M\beta_2$ (CD11b/CD18) integrin (Tang et al., 1997).

PMN also express β_1 integrins, primarily $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, but also very low levels of $\alpha_4\beta_1$ (Gao et al., 1995; Gresham et al., 1996) that participate in PMN adhesion, migration, and phagocytosis. For example, C5a, a cleavage product of the fifth component of complement, and PMA stimulate $\alpha_5\beta_1$-dependent PMN adherence to fibronectin

Address correspondence to Dr. John D. Loike, Department of Physiology and Cellular Biophysics, Columbia University College of Physicians and Surgeons, 630 W. 168th St., Mail hub #22, New York, NY 10032. Tel.: (212) 305-1510. Fax: (212) 305-5775. E-mail: jdl5@columbia.edu

1. *Abbreviations used in this paper:* C3bi, cleaved b fragment of the 3rd complement component; C5a, a fragment of the cleaved 5th complement component; fMLP, formyl methionyl leucyl phenylalanine; HSA, human serum albumin; IL-8, interleukin 8; LTB4, leukotriene B4; PMN, polymorphonuclear leukocytes; Rh-PEG, rhodamine-conjugated polyethylene glycol.

(Bohnsack et al., 1995). Chemoattractant-activated β_1 integrins work in concert with $\alpha_M\beta_2$ (CD11b/CD18) integrins to mediate phagocytosis of particles coated with C3bi by PMN (Pommier et al., 1983; Wright et al., 1984; Brown, 1992). β_1 integrins also mediate chemotaxis of platelet activating factor–stimulated rat PMN (Werr et al., 1998).

We reported previously (Loike et al., 1995) that different chemoattractants specify qualitatively distinct PMN responses when PMN contact specific matrix proteins. For example, PMN stimulated with formyl methionyl leucyl phenylalanine (fMLP) or tumor necrosis factor-α form zones of close apposition on fibrin and do not migrate through fibrin gels, whereas PMN stimulated with leukotriene B4 (LTB4) or interleukin 8 (IL-8) form zones of loose apposition on fibrin and migrate efficiently into and through fibrin gels (Loike et al., 1995). All of these chemoattractants activate PMN β_2 integrins (Diamond and Springer, 1994; Premack and Schall, 1996) and induce PMN to migrate efficiently through three-dimensional matrices composed of Matrigel or collagen I (Loike et al., 1995). Antibodies that block the ligand-binding domains of β_2 integrins inhibit PMN migration through all matrices tested (i.e., collagen I, Matrigel, and fibrin), in response to chemoattractants. Therefore, it seemed unlikely that the different effects of fMLP and LTB4 on PMN chemotaxis through fibrin gels could result from small differences in the effects of these chemoattractants on β_2 integrins.

β_1 integrins regulate the activity of $\alpha_M\beta_2$ integrins on PMN (Brown, 1992) and monocytes (Pommier et al., 1983; Wright et al., 1984), and of $\alpha_{III}\beta_3$ integrins on platelets (Loike et al., 1993). We reasoned that fMLP, but not LTB4, might activate one or more PMN β_1 integrins and that signals generated by the interaction of activated β_1 integrins with ligands on fibrin might affect PMN chemotaxis. To test this hypothesis we examined the effects of fMLP and LTB4 on activation of PMN β_1 integrins, and of antibodies and peptides that block β_1 integrins on fMLP- and LTB4-stimulated PMN adhesion to and migration through fibrin gels. We report here that fMLP, but not LTB4, activates β_1 integrins on PMN, and that the interaction of activated β_1 integrins with fibrin alters the quality of β_2 integrin–dependent adhesion to, and migration through, fibrin gels.

Materials and Methods

Reagents

Rhodamine-conjugated polyethylene glycols of 3.5 kD (Rh-PEG 3.5 kD) and 10 kD (Rh-PEG 10 kD) were prepared as described (Loike et al., 1993, 1995). Sources of antibodies and peptides were as follows: mouse anti-β_1 (P4C10) and the peptides GRGDSP and GRGESP were from GIBCO BRL. Mouse anti–human α_x (LeuM5) was from Organon-Teknika Inc. Mouse anti–human $\alpha_M\beta_2$ (MAC-1) was from Upstate Biotechnology Co. Mouse anti–human α_5 (SAM1), rat anti–human α_6 (GoH3), mouse anti–human α_4 (HP2/1), and mouse anti–human β_3 integrin (SZ21) were from Immunotech. Phycoerythrin-conjugated $F(ab)_2$ anti–mouse IgG was from Jackson ImmunoResearch. Mouse anti-β_3 (PPM6/13) was from Biosource International. Mouse anti-β_3 (MAB1957z) was from Chemicon International. Alexa 488–conjugated $F(ab)_2$ anti–mouse IgG was from Molecular Probes. LTB4, fMLP, PMA, thrombin, and Ficoll-Hypaque were from Sigma Chemical Co. Mouse anti–chicken β_1 integrin (CSAT) and mouse monoclonal anti–human β_1 integrin (AiiB2) were generous gifts from Dr. Clayton Buck (University of California, San Francisco, CA). Mouse mAb 15/7, which recognizes an activation epitope on human β_1 integrins (Bohnsack et al., 1995), was from Athena Neurosciences. Mouse mAb IB4, which blocks the ligand-binding domains of human β_2 integrins (Wright et al., 1983), was a generous gift from Dr. Samuel D. Wright (Merck, Rahway, NJ). PPACK was from Calbiochem-Novabiochem, Matrigel from Becton Dickinson, and collagen I from GIBCO BRL. Purified fibronectin was from Vitex International. Fibrinogen was from American Diagnostica Inc. Fibrinogen uncontaminated by Factor XIII, fibronectin, and vitronectin, a generous gift of Dr. Jeffrey Weitz (MacMaster University, Hamilton, Ontario, Canada), was prepared from fibrinogen obtained from Enzyme Research Labs FIBI. It was first adsorbed with gelatin-agarose to remove fibronectin and then passed over an affinity column to remove Factor XIII. The fibrinogen was precipitated with 25% ammonium sulfate, dialyzed against 150 mM NaCl, 20 mM Tris (pH 7.4), adsorbed with an antibody to human vitronectin linked to Affi-gel, and dialyzed. PAGE analysis showed the resulting fibrinogen to be free of fibronectin or Factor XIII. Western blot analysis revealed no vitronectin (data not shown).

Preparation of Boyden-type Chemotaxis Chambers

Gels, ~1 mm thick, composed of fibrin, Matrigel, or collagen type IV, were formed in cell culture inserts (pore sizes 3 or 8 μm) from Becton Dickinson as described (Loike et al., 1995). Fibrin gels were gently washed with PBS to remove any residual PPACK.

PMN Adhesion and Closeness of Apposition to Fibrin-coated Surfaces

Fibrin/fibrinogen-coated surfaces were prepared as described (Wright et al., 1988; Loike et al., 1992, 1993, 1995) and PMN adhesion was measured by phase-contrast microscopy. Close apposition of PMN to fibrin/fibrinogen-coated surfaces was defined as exclusion of Rh-PEG 10 kD from zones of contact between PMN and fibrin/fibrinogen measured by fluorescence microscopy as described (Loike et al., 1993).

PMN Migration

PMN were prepared as described (Wright et al., 1988) from fresh heparinized blood from healthy adult donors after informed consent. PMN used in these experiments were >95% pure as determined by Wright-Giemsa staining (Wright et al., 1988). 10^6 PMN in 100 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, antibodies, and/or peptides were added to the top and/or bottom compartments in 500 μl of PBSG-HSA. At the end of incubations, 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 a Coulter counter (Loike et al., 1995). Unless otherwise indicated, all values reported are the average of six different samples from at least three independent experiments.

Flow Cytometric Analysis

PMN (10^5 cells/200 μl of PBSG-HSA) were incubated in suspension at 37°C for 30 min in the presence or absence of fMLP (10^{-7} M) or LTB4 (10^{-7} M), transferred to 96-well polystyrene tissue culture microtiter plates (Corning), incubated for 30 min at 4°C in 200 μl PBSG-HSA containing the indicated primary antibody (2 μg/ml), washed three times with PBSG-HSA at 4°C, further incubated for 30 min at 4°C with either Alexa 488–conjugated or phycoerythrin-conjugated rabbit anti–mouse $F(ab')_2$ in 200 μl of PBSG-HSA, washed three times again with PBSG-HSA at 4°C, and resuspended at 4°C in 300 μl PBS containing 2% BSA and 0.3 mg/ml propidium iodide to determine cell viability. The contribution of dead cells (usually <2%) was removed from the final data analysis. The mean fluorescence intensity of 3–5 × 10^3 cells was determined using a Becton Dickinson FACSCalibur®.

Results

PMN Chemotaxis through Matrigel and Fibrin Gels

PMN chemotax through three-dimensional gels composed

of reconstituted basement membrane proteins containing collagen IV, laminin, and fibronectin (Matrigel; Fig. 1), or collagen I (Loike et al., 1995) in response to a gradient of fMLP or LTB4. In contrast, PMN chemotaxis through fibrin gels or plasma clots is dependent upon the specific chemoattractant used. fMLP-stimulated PMN do not migrate through fibrin gels or plasma clots, whereas LTB4-stimulated PMN do (Fig. 2 A; Loike et al., 1995). Checkerboard analyses confirmed that PMN migrate through these gels in response to a chemoattractant gradient (Loike et al., 1995). Placement of equimolar concentrations of both fMLP and LTB4 into the bottom chambers inhibited PMN from migrating through fibrin gels (Fig. 2 A; Loike et al., 1995), confirming that fMLP's effect is dominant over LTB4's effect.

Commercial fibrinogen contains small amounts of fibronectin and vitronectin. To test whether matrix components other than fibrin are responsible for inhibiting migration of fMLP-stimulated PMN through fibrin gels and plasma clots, we performed additional experiments using fibrin gels formed from purified fibrinogen that contained no detectable fibronectin, plasminogen, Factor XIII, or vitronectin. PMN stimulated with LTB4, but not with fMLP, migrated through gels formed from purified fibrinogen (Fig. 2 B). Moreover, collagen I gels (60 μg/insert) each containing 10 μg of purified fibronectin did not affect the migration of either fMLP- or LTB4-stimulated PMN, whereas the addition of fibrinogen to such gels blocked migration of fMLP-stimulated PMN (data not shown). These results are consistent with reports (Asakura et al., 1997; Farrell and al-Mondhiry, 1997; Suehiro et al., 1997; Miettinen et al., 1998) that fibrin(ogen) contains sequences that are ligands for β_1 integrins, and confirm that fibrin is the matrix component that inhibits migration of fMLP-stimulated PMN.

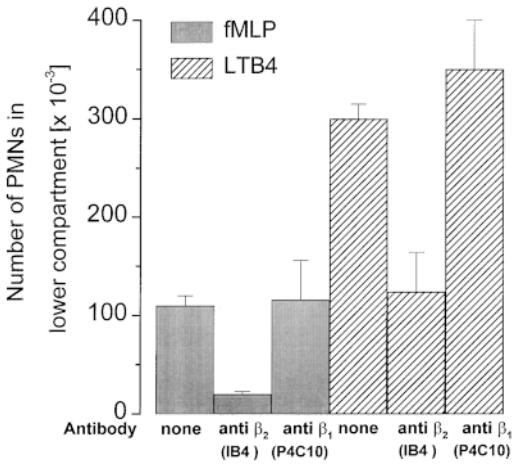

Figure 1. Effects of mAbs against β_1 and β_2 integrins on PMN chemotaxis through inserts precoated with Matrigel. 0.1 ml PBS containing 22 μg of Matrigel was placed into each culture insert (pore size = 8 μm) and allowed to gel at room temperature for 24 h. 10^6 PMN in 250 μl PBSG-HSA were placed in the upper compartment of each insert. 10^{-7} M LTB4 or 10^{-7} M fMLP in 250 μl of PBSG-HSA was added to the bottom compartment. Where indicated, PMN were preincubated at 4°C for 30 min in PBSG-HSA containing 2 μg/ml of either anti-β_1 or anti-β_2 antibodies before adding the cells to the upper compartment of the inserts. Inserts were incubated for 6 h at 37°C, at which time the medium in each lower compartment was collected and its PMN content was determined using a Coulter counter. Reported are the average number ± SEM of PMN that migrated into the lower compartment from at least two samples in each of three independent experiments.

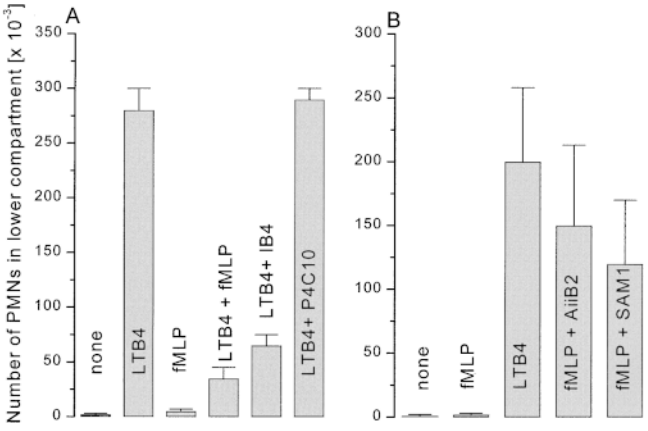

Figure 2. LTB4 but not fMLP promotes PMN migration through fibrin gels. Fibrin gels were prepared from commercial fibrinogen (A) or from purified fibrinogen (B) and formed on top of filters (8-μm pores) in tissue culture inserts as described in Materials and Methods. 10^6 PMN were added to the upper chamber and 5×10^{-8} M fMLP or 10^{-7} M LTB4 was added to either the lower or upper chamber as indicated. Where indicated, PMN were preincubated with antibodies against β_1 (AiiB2) or against $\alpha_5\beta_1$ (SAM1) integrins at 2 μg/ml for 30 min at 4°C and the mixture was added to the upper chamber. Inserts were incubated at 37°C for 6 h, at which time the number of cells in the lower chamber was determined using a Coulter counter.

Effects of Antibodies against β_1 and β_2 Integrins on PMN Chemotaxis through Fibrin Gels and Matrigel

To examine the roles of β_1 and β_2 integrins in PMN migration through Matrigel (Fig. 1), or fibrin (Figs. 2 A and 3), we added antibodies that block β_1 or β_2 integrins to the upper compartment of Matrigel or fibrin-coated inserts together with PMN and measured the number of PMN that migrated into the lower compartment in response to fMLP or LTB4. As expected, mAb IB4, directed against β_2 integrins (Wright et al., 1983), blocked PMN migration through Matrigel (Fig. 1) or fibrin gels (Fig. 2 A) in response to LTB4. Antibody IB4 also blocked fMLP-stimulated PMN migration through Matrigel (Fig. 1), and did not alter fMLP's inhibitory effect on PMN chemotaxis through fibrin gels (data not shown). These results are consistent with previous reports (Diamond and Springer, 1994; Springer, 1995; Premack and Schall, 1996) that anti–β_2 integrin antibodies block PMN migration through endothelia and through gels formed by a variety of extracellular matrix proteins.

In contrast, mAbs AiiB2 (Bohnsack et al., 1990) and P4C10 (Carter et al., 1990), which block the common β chain of β_1 integrins (CD29), had no effect on fMLP- or LTB4-stimulated chemotaxis through Matrigel (Fig. 1), or on LTB4-stimulated PMN migration through fibrin gels

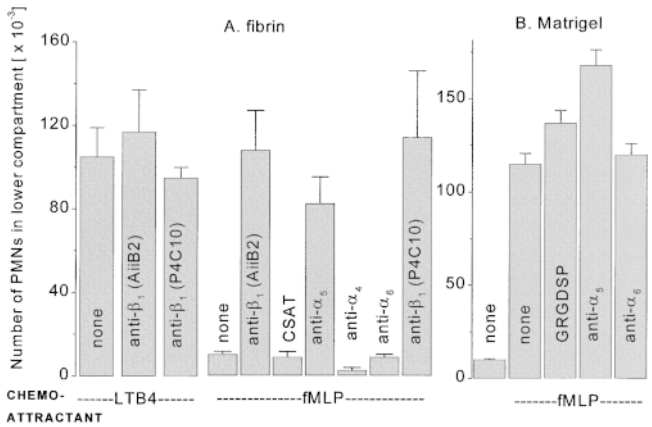

Figure 3. Effects of anti–β_1 and anti–β_2 integrins on PMN chemotaxis through fibrin-coated inserts or Matrigel-coated inserts. Inserts were prepared as described in Fig. 1. Where indicated, PMN were preincubated for 30 min at 4°C with 2 μg/ml of one of the following mAbs: mouse anti-β_1 (P4C10), mouse anti–human α_4 (HP2/1), mouse anti–human α_5 (SAM1), or rat anti–human α_6 (GoH3), or with 1 mg/ml of GRGDSP peptide. The mixture was then added to the upper compartment of fibrin-coated inserts. 10^{-7} M LTB4 or 5×10^{-8} M fMLP was placed in the lower compartment, and the inserts were incubated at 37°C for 6 h. The number of PMN in the lower compartment was assayed as described in Fig. 1.

(Fig. 2 A). However, these same anti–β_1 chain antibodies reversed fMLP's inhibitory effect on PMN chemotaxis through fibrin (Fig. 3 A).

Control experiments showed that CSAT (Lallier and Bronner-Fraser, 1991), a mAb that binds to chicken but not human β_1 integrins, SZ21, an antibody against β_3 integrins (Lawson and Maxfield, 1995), and PM6/13, another antibody against β_3 integrins (Patel et al., 1998), did not alter the inhibitory effect of fMLP on PMN migration through fibrin gels (Fig. 3 A and data not shown). These antibodies also did not affect migration of LTB4-stimulated PMN through fibrin gels (data not shown).

Among the antibodies directed against the α chains of β_1 integrins, only those directed against α_5 chains were effective in reversing fMLP's inhibitory effect on PMN migration through fibrin gels (Figs. 2 B and 3 A). Neither antibodies against α_4 chains nor antibodies against α_6 chains of β_1 integrins affected migration of fMLP- or LTB4-stimulated PMN through fibrin gels (Fig. 3 A) or Matrigel (Fig. 3 B and data not shown).

To confirm that β_1 integrins directly interact with fibrin(ogen), we examined the effects of anti–β_1 integrins on the migration of fMLP-stimulated PMN through gels formed of purified fibrinogen, lacking detectable levels of fibronectin, vitronectin, plasminogen, or Factor XIII. Both antibodies directed against β_1 and α_5 chains of β_1 integrins (Fig. 2 B) reversed fMLP's inhibitory effect on chemotaxis through these gels. These results are consistent with reports (Asakura et al., 1997; Farrell and al-Mondhiry, 1997; Suehiro et al., 1997; Miettinen et al., 1998) that fibrin(ogen) contains sequences that are ligands for β_1 integrins.

Table I. Effect of RGD-containing Peptides on fMLP- and LTB4-stimulated PMN Chemotaxis through Fibrin Gels

Additions	Chemoattractant	PMN in lower compartment ($\times 10^{-3}$)
None	fMLP	3 ± 1
G**RGD**SP	fMLP	50 ± 5
G**RGE**SP	fMLP	2 ± 1
None	LTB4	135 ± 30
G**RGD**SP	LTB4	170 ± 50
G**RGE**SP	LTB4	110 ± 40

10^6 PMN were preincubated for 30 min at 4°C with PBSG-HSA alone or containing 1 mg/ml GRGDSP or GRDESP before adding the mixture to the upper compartment of chemotaxis chambers. LTB4 (10^{-7} M) or fMLP (10^{-7} M) was added to the lower compartment, the chambers were incubated at 37°C for 6 h, and the number of PMN that migrated into the lower compartment was determined, all as described in Fig. 1. Values represent the average ± SEM of triplicate samples from three experiments.

The peptide GRGDSP blocks the interaction of β_1 integrins with RGD ligands on matrix proteins (Pierschbacher and Ruoslahti, 1987). Like antibodies against β_1 integrins, addition of GRGDSP peptide to the medium allowed fMLP-stimulated PMN to migrate through fibrin gels (Table I). Control experiments showed that GRGESP peptide, which does not block binding of β_1 integrins to fibronectin or other RGD-containing matrix proteins (Pierschbacher and Ruoslahti, 1987), did not reverse the inhibitory effect of fMLP on PMN migration through fibrin gels (Table I). Neither peptide affected the number of PMN that migrated through fibrin in response to LTB4 (Table I) or through Matrigel in response to fMLP (Fig. 3 B).

Effects of Antibodies against β_1 and β_2 Integrins on Adhesion of Chemoattractant-stimulated PMN to Fibrin

PMN were incubated in control medium or in medium containing fMLP or LTB4 and allowed to adhere to fibrin-coated 96-well plates. In the absence of chemoattractant <1% PMN adhered to fibrin (data not shown). Over 40% of fMLP-stimulated PMN and ~50% of LTB4-stimulated PMN adhered to fibrin. mAb IB4, which blocks the ligand-binding sites of three different β_2 integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$, and $\alpha_X\beta_2$; Wright et al., 1983; Loike et al., 1991), inhibited adhesion of fMLP- or LTB4-stimulated PMN to fibrin by 75–80% (Fig. 4). In contrast, mAb AiiB2, which blocks the ligand-binding sites of β_1 integrins (Bohnsack et al., 1990), had no significant effect on the number of fMLP-stimulated PMN that adhered to fibrin, and enhanced by ~25% adhesion of LTB4-stimulated PMN to fibrin (Fig. 4). These experiments show that β_2 integrins are the primary PMN surface receptors that mediate adhesion of chemoattractant-stimulated PMN to fibrin.

fMLP, but Not LTB4, Activates PMN $\alpha_5\beta_1$ Integrins

The findings presented above indicate that β_1 integrins, and specifically $\alpha_5\beta_1$ integrins, mediate the qualitatively distinct effects of fMLP and LTB4 on PMN adhesion to, and migration through, fibrin gels. To determine whether fMLP and LTB4 differentially affect the activation of β_1 integrins we used mAb 15/7, which recognizes a confor-

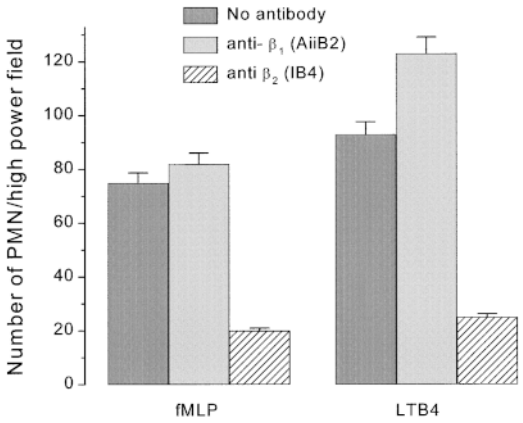

Figure 4. Effect of anti-β_1 and anti-β_2 antibodies on adhesion of fMLP- or LTB4-stimulated PMN to fibrin-coated surfaces. 5×10^3 PMN in 5 μl PBSG-HSA containing 10^{-7} M fMLP or 10^{-7} M LTB4 were allowed to settle for 30 min at 4°C in the presence or absence of the indicated antibody (10 μg/ml) onto each well of a 96-well plate that had been precoated with fibrin. Then, cells were warmed to 37°C for 15 min. The plates were washed, fixed, and counted as described (Wright et al., 1988).

mationally determined epitope on activated β_1 integrins (Bohnsack et al., 1995). PMN incubated for 30 min with fMLP exhibited a 10–22-fold increase in binding of mAb 15/7 (Fig. 5 J), compared with unstimulated PMN (Fig. 5 B), whereas PMN incubated for the same length of time with LTB4 (Fig. 5 F) showed little change over unstimulated PMN (Fig. 5 B) with respect to binding of mAb 15/7. Control experiments showed that surface expression of β_1 integrins was stimulated approximately twofold by LTB4 (Fig. 5 G), and approximately threefold by fMLP (Fig. 5 K), whereas β_2 integrin surface expression was stimulated approximately fivefold by LTB4 (Fig. 5 H) and approximately ninefold by fMLP (Fig. 5 L). Other studies showed that the extent of expression of the epitope for antibody 15/7 on β_1 integrins was dependent upon the dose of fMLP used to stimulate the PMN, and that 5×10^{-6} M fMLP induced maximal expression of this epitope (not shown). In contrast, LTB4 concentrations 10–50-fold higher (i.e., 10^{-6} to 5×10^{-6} M) than those used in the experiments described in Fig. 5 did not increase expression of the 15/7 epitope on β_1 integrins (data not shown).

Effects of Antibodies against β_1 Integrins on Closeness of Apposition of fMLP- and LTB4-stimulated PMN to Fibrin

We have used exclusion of Rh-PEG 10 kD from zones of contact between chemoattractant-stimulated PMN and fibrin-coated surfaces as a measure of the closeness of apposition of PMN to the underlying substrate (Loike et al., 1995). Previously, we reported an inverse correlation between the formation of zones of close apposition between chemoattractant-stimulated PMN and fibrin gels and the capacity of PMN to migrate through these gels (see Fig. 7 in Loike et al., 1995). In the present experiments we used exclusion of Rh-PEG 10 kD to test whether antibodies and peptides that block β_1 integrins, and that facilitate migration of fMLP-stimulated PMN through fibrin gels (Figs. 2 and 3 A and Table I), affect the closeness of apposition of these cells to fibrin. Antibodies against the β chain of β_1 integrins, or against the α_5 chain of $\alpha_5\beta_1$ integrins (not shown), reduced the percentage of fMLP-stimulated PMN that excluded Rh-PEG 10 kD from zones of contact with fibrin from 80% to 20–30% (Fig. 6), and reduced the percentage of LTB4-stimulated PMN that excluded Rh-PEG 10 kD from these contact zones from 20% to <2% (Fig.

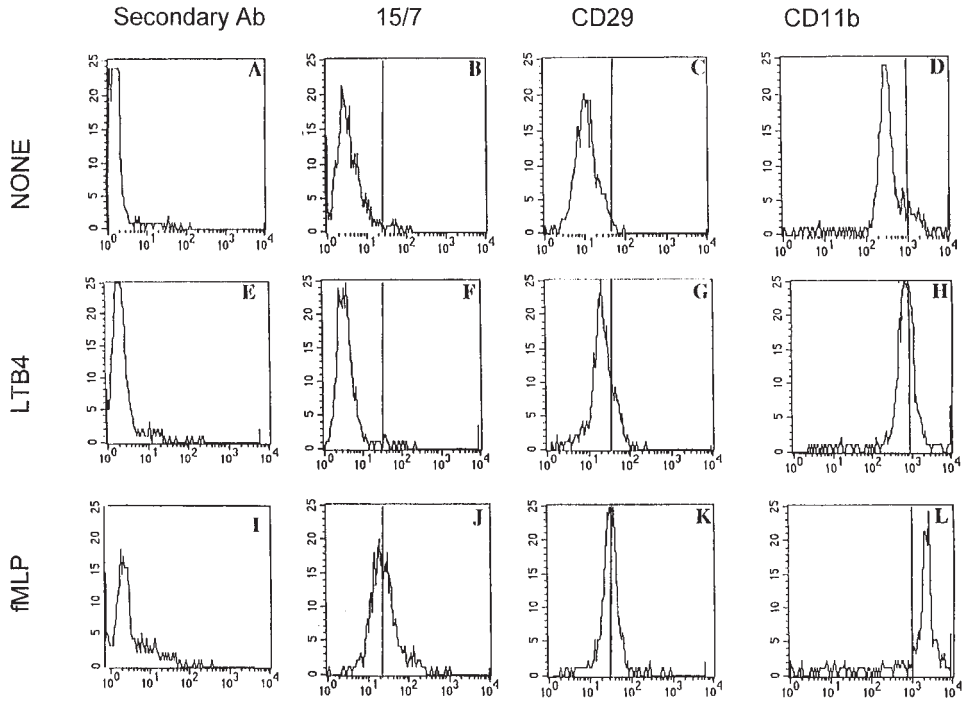

Figure 5. Effect of fMLP and LTB4 on activation of PMN β_1 integrins. Fluorescence intensity of PMN incubated as described in Materials and Methods with Alexa 488–conjugated rabbit $F(ab')_2$ anti–mouse IgG alone (A, E, and I), with mAb 15/7 that recognizes an activation epitope on β_1 integrins followed by Alexa 488–conjugated $F(ab')_2$ anti–mouse IgG (B, F, and J), with mAb CD29 that recognizes the β chain of human β_1 integrins followed by Alexa 488–conjugated $F(ab')_2$ anti–mouse IgG (C, G, and K), or with a mAb that recognizes $\alpha_M\beta_2$ (CD11b/CD18) followed by Alexa 488–conjugated rabbit $F(ab')_2$ anti–mouse IgG (D, H, and L). Unstimulated PMN (A–D), LTB4-stimulated PMN (E–H), fMLP-stimulated PMN (I–L). fMLP and LTB4 were each used at 10^{-7} M.

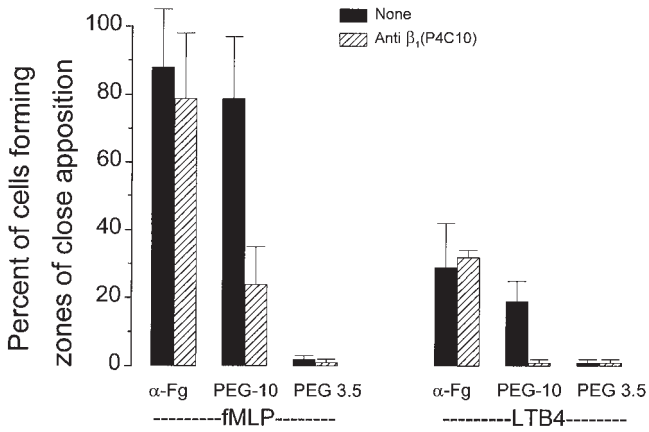

Figure 6. Effect of anti–β_1 integrin antibodies on formation of zones of close apposition between PMN and fibrin-coated surfaces. 5×10^3 PMN in 30 μl PBSG-HSA containing 10^{-7} M fMLP, or 10^{-7} M LTB4, were allowed to adhere to each fibrin-coated spot of multispot slides in the presence or absence of P4C10 anti–β_1 integrin antibody (2 μg/ml) for 30 min at 37°C. The preparations were further incubated at room temperature for 30 min with either fluorescein-conjugated antifibrinogen IgG (Loike et al., 1995), Rh-PEG 10 kD, or Rh-PEG 3.5 kD (Loike et al., 1992, 1993, 1995) and examined by epifluorescence microscopy. A zone of close apposition is defined as a region between adherent PMN and the underlying matrix from which fluorophore-conjugated probe is excluded. Values are expressed as the percentage of adherent PMN that forms such zones. Note that Rh-PEG 3.5 kD stained the surfaces uniformly, confirming the presence of fibrin underneath all adherent PMN and showing that molecules of 3.5 kD penetrate zones of close apposition.

6). These experiments together with those shown in Fig. 5 show there is a direct correlation between the capacity of fMLP or LTB4 to activate PMN β_1 integrins and the capacity of these chemoattractants to promote close apposition between PMN and fibrin (as measured by exclusion of Rh-PEG 10 kD), and to inhibit PMN migration through fibrin gels (Fig. 3 A).

Effects of Phorbol Esters on PMN Adhesion and Migration

Tumor-promoting phorbol esters, like ligands that bind to PMN and macrophage fibronectin receptors, activate $\alpha_M\beta_2$ (CD11b/CD18) for phagocytosis of C3bi-coated particles (Wright and Silverstein, 1982), and promote formation of zones of close apposition between phorbol ester–stimulated PMN and fibrinogen-coated surfaces (Table II). However, phorbol ester–stimulated PMN do not migrate into fibrin gels, even when treated with antibodies against $\alpha_5\beta_1$ integrins (data not shown). These findings suggested that phorbol esters activate $\alpha_M\beta_2$ integrins for close apposition to fibrin independently of β_1 integrins. To test this prediction, PMN were incubated with or without antibodies against β_1 integrins, allowed to adhere to fibrin- or fibrinogen-coated surfaces in medium containing PMA, and then were incubated with Rh-PEG 10 kD. 77% of PMA-treated PMN formed zones of close apposition on fibrin even when they had been treated with antibodies against

Table II. Effect of Antibodies against β_1 or β_2 Integrins on Formation of Zones of Close Apposition to Fibrin by PMA-stimulated PMN

Additions	Percent of PMN forming zones of close apposition	
	Fibrin-coated surfaces	Fibrinogen-coated surfaces
None	78 ± 6	94 ± 2
Anti-β_1	77 ± 3	95 ± 1
Anti-β_2	12 ± 4	14 ± 5

5×10^3 PMN stimulated with PMA (300 ng/ml) in 30 μl PBSG-HSA were allowed to adhere to each fibrin- or fibrinogen-coated spot of multispot slides in the presence or absence of P4C10 anti–β_1 integrin antibody (2 μg/ml) for 30 min at 37°C. The preparations were further incubated at room temperature for 30 min with Rh-PEG 10 kD, and examined by epifluorescence microscopy. A zone of close apposition is defined as described in Fig. 6. Values represent the average ± SEM of triplicate samples from three experiments.

β_1 integrins. In contrast, <15% of the PMA-stimulated PMN that adhered to these surfaces formed zones of close apposition when treated with antibodies against β_2 integrins (Table II). This experiment shows that when suitably activated, β_2 integrins are capable of mediating close apposition between PMN and fibrin-coated surfaces in the absence of β_1 integrin ligation.

Discussion

The different effects of fMLP and LTB4 on PMN adhesion to and chemotaxis through fibrin gels appear to be a consequence of qualitative differences in the effects of these chemoattractants on the activity of β_1 integrins. That is, fMLP activates β_1 integrins (Fig. 5 and Table III), stimulates PMN to adhere closely to fibrin(ogen) (Fig. 6 and Table III; Loike et al., 1995), and inhibits PMN chemotaxis through fibrin gels (Fig. 2 and Table III; Loike et al., 1995). In contrast, LTB4 neither activates β_1 integrins (Fig. 5 and Table III) nor induces PMN to adhere closely to fibrin(ogen) (Fig. 6 and Table III; Loike et al., 1995), and stimulates PMN to migrate through fibrin gels (Fig. 2 and Table III; Loike et al., 1995). To our knowledge, this is the first demonstration that signals initiated by two chemically distinct chemoattractants with their respective seven membrane spanning/heterotrimeric G protein–coupled receptors exert different effects on the activation state of a specific β_1 integrin, and regulate PMN migration.

Fibrin(ogen)-containing Matrices Exert a Specific Effect

As shown in Fig. 2, fibrin(ogen) is unique among the matrix and plasma proteins tested in arresting the migration of fMLP-stimulated PMN. This is particularly notable in the case of fibronectin, a well-recognized ligand for $\alpha_5\beta_1$ integrins. The failure of fibronectin to induce migration arrest suggests that fibrin(ogen) has heretofore unrecognized properties, independent of its ability to bind $\alpha_5\beta_1$ integrins, that are important in its ability to cause migration arrest.

Relationship between Closeness of Apposition, Tightness of Adhesion, and Cell Migration

DiMilla et al. (1993) and Palecek et al. (1997) reported

Table III. Summary of Results

		Chemoattractant*						
		fMLP			LTB4			
Assay	mAb added:	None	Anti-β_1	Anti-β_2	None	Anti-β_1	Anti-β_2	None
Adhesion to fibrin*		>40%	>40%	<10%	~50%	~66%	<10%	<5%
Chemotaxis		−	+	−	+	+	−	−
Exclusion Rh-PEG (10 kD)		80%	20%	<5%	20%	2%	<5%	<5%
Binding mAb 15/7		~10×	ND bkg	ND	bkg	ND	ND	bkg

*Percent of control. About 40% and 60% of the added PMN adhered to the fibrin-coated surfaces in response to fMLP and LTB4, respectively. ND, not done; bkg, background.

that smooth muscle cells migrate optimally on fibronectin-coated surfaces when their integrins bind to these surfaces at intermediate strengths. Weber et al. (1996) reported an inverse correlation between the strength of adhesion of chemokine-stimulated monocytes to surfaces coated with the 120-kD RGD-containing fibronectin fragment and the capacity of these cells to migrate across filters coated with this fibronectin fragment. The findings of Keller et al. (1979) and of Wilkinson et al. (1984), and those reported in Fig. 6, demonstrate an inverse correlation between closeness of apposition of PMN to surfaces coated with proteins that express ligands for PMN receptors and the ability of PMN to migrate on or through matrices containing these proteins. Thus, it seems likely that loose versus close apposition between cells and matrix protein–coated substrates reflects weak versus strong adhesion, respectively, between the cells and the substrate.

PMA Bypasses β_1 Integrins in Stimulating PMN to Adhere Closely to Fibrin

Antibodies against β_2 integrins reduced adhesion, inhibited close apposition between PMA-stimulated PMN and fibrin (Table II), and blocked PMN migration through fibrin (data not shown). Antibodies against β_1 integrins had no effect on any of these parameters (Table II and data not shown). These results demonstrate that the interaction of activated β_2 integrins with fibrin is both required and sufficient for PMA-stimulated PMN to form zones of close apposition on fibrin (Table II), and that PMA bypasses the requirement for engagement of activated β_1 integrins by matrix proteins for PMN to form zones of close apposition on fibrin.

Pathways by Which fMLP and LTB4 Activate β_1 and β_2 Integrins

Although the signal transduction pathways by which chemoattractants regulate PMN β_1 and β_2 integrins remain to be elucidated, our findings lead us to make three suggestions regarding the organization of these pathways.

First, antibodies that activate β_1 integrins do not promote adhesion of unstimulated PMN to fibrin, or inhibit LTB4-stimulated chemotaxis of PMN through fibrin gels (unpublished data). These results suggest that signals initiated by both fMLP receptors and activated β_1 integrins are required to inhibit chemotaxis of fMLP-stimulated PMN through fibrin gels.

Second, the finding that fMLP and PMA have similar effects on PMN adhesion to and migration through fibrin gels might suggest that the interaction of activated β_1 integrins of fMLP-stimulated PMN with fibrin activates protein kinase C, and that this is the mechanism by which fMLP signals β_2 integrins to bind closely to fibrin. However, Laudanna et al. (1996) reported that calphostin C, a protein kinase C inhibitor, blocks adhesion of PMA-stimulated, but not of fMLP-stimulated, mouse lymphocytes transfected with fMLP receptors, to VCAM-1–coated surfaces. (Adhesion of chemokine-stimulated lymphocytes to VCAM-1 is mediated by activated $\alpha_4\beta_1$ integrins.) Laudanna et al. (1996) identified rho as a key participant in fMLP- and IL-8–mediated activation of $\alpha_4\beta_1$ integrins in mouse lymphocytes. This finding suggests to us that rho acts downstream of $G\alpha_i$ in activating β_1 integrins. The report of Caron and Hall (1998) that rho participates in coupling CR3 (CD11b/CD18) to the actin cytoskeleton suggests that rho also affects β_2 integrin–mediated functions. Whether PMN LTB4 receptors activate rho is unknown and should be investigated.

Third, binding of fMLP to its receptor activates $G\alpha_i$ (Laudanna et al., 1996). The specific $G\alpha$ activated by LTB4 in PMN has not been reported. Pertussis toxin, which inactivates $G\alpha_i$, blocks most effects of LTB4 and of fMLP on human PMN. Thus, the finding that fMLP activates β_1 integrins (Fig. 5 J) while LTB4 does not (Fig. 5 F) suggests that binding of LTB4 to its receptor activates $G\alpha$ subunits other than, or in addition to, $G\alpha_i$ and that this difference in $G\alpha$ subunit utilization is responsible for the divergent effects of fMLP and LTB4 on β_1 integrin activation (Fig. 5), and on closeness of PMN adhesion to fibrin (Fig. 6). Indeed, Arai and Charo (1996) have shown differential utilization of $G\alpha$ subunits after MCP-1 or IL-8 stimulation of MCP-1 or IL-8 receptor transfected HEK293 cells, and Yokomizo et al. (1997) have demonstrated that pertussis toxin treatment does not ablate Ca^{2+} increases stimulated by LTB4 in LTB4 receptor-bearing CHO cells.

Proposed Mechanisms by Which fMLP Inhibits PMN Chemotaxis through Fibrin Gels

Our studies suggest at least three distinct mechanisms by which fMLP could inhibit PMN migration through fibrin gels. First, the combined strengths of adhesion of activated β_1 and β_2 integrins to fibrin could be sufficient to immobilize PMN on fibrin. Our unpublished finding that antibodies that activate β_1 integrins do not inhibit migration of LTB4-stimulated PMN through fibrin gels casts doubt on this combined-strength-of-adhesion hypothesis as an ex-

planation for the inhibitory effect of fMLP on PMN chemotaxis through fibrin.

Second is the possibility that binding of fMLP or LTB4 to its cognate receptors directly and differentially activates β_2 integrins for strong or weak adhesion, respectively. According to this hypothesis, fMLP-activated β_1 integrins play no role in inhibiting chemotaxis of fMLP-stimulated PMN through fibrin. However, since activated β_1 integrins mediate outside-in signaling, RGD peptides and antibodies against β_1 integrins reverse fMLP's inhibitory effect on PMN migration through fibrin by stimulating β_1 integrins to signal trans-dominant negative (Diaz-Gonzalez et al., 1996) effects on β_2 integrins. Against this hypothesis are the findings that antibodies against the α_6 chains of β_1 integrins (Fig. 3), and antibodies that activate β_1 integrins (unpublished data), do not reverse fMLP's inhibitory effect on PMN chemotaxis through fibrin.

Third, and we think most likely, is that the capacity of fMLP to promote close adhesion to, and to block migration through, fibrin gels is mediated by a cascade of signals (diagrammed in Fig. 7), in which the interaction of activated β_1 integrins with the fibrin matrix causes trans-dominant activation of β_2 integrins. This mechanism is consistent with previous studies (Pommier et al., 1983; Wright et al., 1984; Brown, 1992) showing that interaction of PMN or macrophages with RGD-containing matrix proteins activates $\alpha_M\beta_2$ integrins for phagocytosis of C3bi-coated particles.

As shown in Fig. 7, we suggest that the interaction of LTB4 or fMLP with their respective PMN receptors generates a "common" signal that activates β_2 integrins for loose adhesion to fibrin (Fig. 7, A and B, and D and E, respectively). In addition, we propose that fMLP receptors (Fig. 7 C) also signal activation of $\alpha_5\beta_1$ integrins (Figs. 5 J and 7 E). We further suggest that binding of activated $\alpha_5\beta_1$ integrins to fibrin matrices clusters these integrins, thereby generating an outside-in signal that activates β_2 integrins (Fig. 7 F), for close apposition between PMN and fibrin-coated substrates (Fig. 7 G).

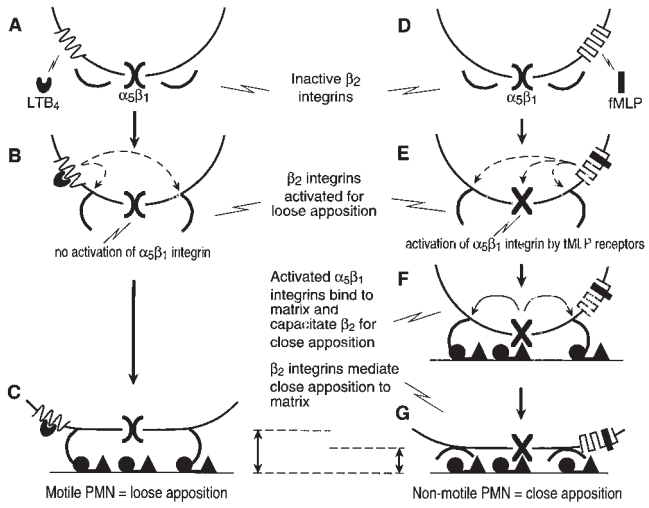

Figure 7. Proposed mechanism by which fMLP and LTB4 regulate β_1 and β_2 integrins, and thereby regulate PMN chemotaxis through fibrin gels. See Discussion for description.

Close apposition reflects tight adhesion (Keller et al., 1979; Wilkinson et al., 1984; DiMilla et al., 1993; Palecek et al., 1997), presumably mediated by the coupling of β_2 integrins to the cytoskeleton. We do not know whether tight adhesion causes, or is merely associated with, cessation of migration. In either case, PMN cease migrating (Figs. 2 A and 3 A, and Table III). We propose that antibodies and peptides that block the interaction of activated $\alpha_5\beta_1$ integrins with fibrin (Figs. 2 and 3, and Table I) inhibit these outside-in signals, thereby blocking trans-dominant activation of β_2 integrins for close apposition to fibrin (Fig. 6 and Table II) and allowing PMN to migrate through fibrin.

The interaction of LTB4 with its receptor also generates a signal that stimulates β_2 integrins for loose apposition. However, LTB4 does not activate β_1 integrins (Fig. 5 F). Therefore, these β_1 integrins do not bind to the matrix, do not generate outside-in signals, and therefore do not initiate trans-dominant activation of β_2 integrins for close apposition (Fig. 7, A–C), or cessation of migration.

What Characterizes the Sessile State?

Further work is needed to determine whether cessation of migration is merely a function of strong adhesion between PMN and fibrin or whether it reflects reorganization of the PMN cytoskeleton as observed by Dustin et al. (1997) in antigen-sensitized T lymphocytes. They found that these cells become immobilized when they encounter MHC class II molecules containing a peptide antigen recognized by the T lymphocytes' antigen receptors. They identified changes in microtubule organization of these sessile T lymphocytes that distinguish them from their randomly migrating brethren. We suspect that PMN that adhere to fibrin after fMLP stimulation will exhibit similar changes in cytoskeletal organization.

Why Are There So Many Different Chemoattractants for PMN?

Our findings suggest that the availability of many different chemoattractants (e.g., fMLP, LTB4, IL-8, C5a, etc.) serves two complementary functions. First, they provide redundancy, thereby assuring that pathogenic microbes are detected rapidly by the innate immune system. Second, they reflect the need to direct PMN to different tissue sites and to prepare them for interactions with many different types of ligands.

Chemoattractant-encrypted Stop Signals Provide a Gradient-independent Mechanism for Leukocyte Accumulation at Specific Anatomic Sites

Our findings also suggest an alternative to the notion that leukocyte accumulation at a specific anatomic site in vivo requires the presence of a gradient of chemoattractant/chemokine emanating from that site. While there is no doubt that gradients of chemoattractants/chemokines are formed in vitro (Keller et al., 1979; Wilkinson et al., 1984; Huber et al., 1991; Campbell et al., 1996, 1997; Foxman et al., 1997; Palecek et al., 1997), they may be difficult to maintain in vivo in the face of the perturbing effects of muscular contraction and variations in blood and lymph flow.

Leukocytes in the vascular system begin to enter specific

tissue compartments when they encounter a chemoattractant/chemokine. We suggest that once within this tissue compartment leukocytes migrate randomly in response to a relatively uniform concentration of matrix-bound chemoattractant/chemokine. When in the course of this random walk they encounter extracellular matrix proteins or cells that express ligands for a specific activated β_1 integrin, they adhere strongly and become sessile. By regulating activation of specific receptors and adhesive strengths, concentrations of chemoattractants/chemokines well below those required to saturate or desensitize chemoattractant/chemokine receptors can mediate a stochastic process by which leukocytes accumulate at specific anatomic sites and form highly ordered structures (e.g., granulomas, germinal centers). According to this model, leukocytes accumulate at specific anatomic sites by a process that is similar in principle to the accumulation of flies on fly paper.

Foxman et al. (1997) showed that multiple chemoattractants/chemokines can work in combination to elicit migration patterns that cannot be achieved by a single chemoattractant/chemokine. The mechanisms we and they have described are complementary. These mechanisms are likely to be of special importance within tissue compartments where overlapping fields of chemoattractants/chemokines/cytokines surely occur, and where cells migrate in stepwise fashion from one anatomic site to another (e.g., T cell movement in lymph nodes from T cell–rich paracortical zones to germinal centers; Garside et al., 1998), PMN accumulation at foci of bacterial infection, or of immune-complex deposition (Wilkinson et al., 1984). The essential point of the findings reported here is that by endowing leukocytes, and probably all migrating cells, with a modest number of receptors for different chemoattractants, chemokines, and cytokines, nature has made optimal use of instructive and selective mechanisms to achieve a level of organizational specificity that would otherwise require substantially more genetic information.

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