Promotion of Neutrophil Chemotaxis Through Differential Regulation of β_1 and β_2 Integrins[1]

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Migration of neutrophils requires sequential adhesive and deadhesive interactions between β_1 and β_2 integrins and components of the extracellular matrix. Prompted by reports that describe interaction of soluble β-glucan with the β_2 integrin Mac-1, a role for β-glucan in regulation of integrin-mediated migration was investigated. Neutrophil migration in response to fMLP was assessed using an agarose overlay method with slides precoated with fibronectin (Fn) ± β-glucan. On Fn, random migration in excess of directed migration was observed. In contrast, migration on Fn + β-glucan was directional, with marked diminution of random migration. This conversion of random to directed migration was seen neither when Fn was supplemented with alternative polysaccharides nor when β-glucan was applied to other components of the extracellular matrix. This effect of β-glucan was shown to be cation dependent and to be effected by Arg-Gly-Asp-containing peptides consistent with an integrin-mediated event. mAb inhibition studies demonstrate that β-glucan effects this shift toward directed migration through suppression of migration mediated by Mac-1 and very late Ag 5 and enhancement of very late Ag 3-mediated migration. Adhesion assays suggest that the prochemotactic influence of β-glucan is due, in part but not entirely, to modulation of PMN adhesion to Fn. In summary, these data support a novel role for β-glucan in regulation of β_1- and β_2-mediated neutrophil migration on Fn. ***The Journal of Immunology,*** **1999, 162: 6792–6799.**

Localization of circulatory polymorphonuclear leukocytes (PMN)[3] to extravascular sites of inflammation is a function of repeated adhesive and deadhesive events. The sequential processes of PMN rolling, adhesion, and transendothelial migration are facilitated by cell surface adhesion receptors referred to as selectins and integrins (1). Following extravasation, PMN migrate toward a source of inflammation in response to locally elaborated chemotaxins and cytokines (2). Stimulated by a chemotactic gradient, PMN traverse the extracellular matrix (ECM) by way of transient interactions between integrin receptors and components of the ECM that serve as adhesive ligands. Activation of specific integrins through ligand binding has been shown to augment cell adhesion and precipitate reorganization of the actin cytoskeleton (3). Polarization of the migrant cell ensues, with development of lamellopodia and filopodia (4). Traction at the leading edge of the cell develops through integrin engagement, followed by translocation of the cell over the adherent segment of plasma membrane. Having been shifted to the rear of the cell, the integrin then releases its substrate, permitting the cell mass to advance (5). Integrin receptors reported to contribute to the process of neutrophil locomotion include members of the β_2 (LFA-1 and Mac-1) (6–8) and β_1 subfamilies (VLA-4, -5, and -6) (9, 10).

The mechanisms that regulate the recruitment of extravasated PMN to foci of inflammation are not well established. Regulation of integrin function by adhesive substrates offers a mechanism for local control of migrant cells. Within the assembled framework of the ECM, binding sites for integrins have been identified on collagen (11), laminin (12), and fibronectin (Fn) (13) among others. Because cell migration is the product of sequential adhesive and deadhesive events, those ligands that permit efficient forward attachment and rearward detachment should theoretically serve as optimal substrates.

Mac-1 (CD11b/CD18, CR3, Mo-1) is a multifunctional receptor most prominently expressed on myeloid and NK cells (14, 15). Structurally classified as an adhesion molecule, with particular relevance to cell-mediated interaction with ECM, recent investigations identify Mac-1 participation in a variety of cellular functions. Activities facilitated by Mac-1 include homotypic aggregation (7), adhesion (16), migration (8), and binding and phagocytosis of opsonized microbial pathogens (17, 18). The versatility of Mac-1 is related to its ability to bind an array of soluble and insoluble ligands, including zymosan (19), fibrinogen (20), ICAM-1 (21), iC3b (22), factor X (23), and heparin sulfate (24). Mac-1 is unique among the β_2 integrins in that it also contains a lectin site capable of interacting with *N*-acetyl-D-glucosamine (19), glucose, and possibly mannose (25) residues. Moreover, recent reports describe regulation of Mac-1 function through binding of soluble β-glucan to the lectin site (26). Specifically, β-glucan has been shown to induce cytotoxicity in NK cells for iC3b-opsonized target cells that were otherwise resistant to killing.

The β-glucans are a class of long chain polymers of glucose in $\beta_{1,3}$ linkages that comprise the cell wall of yeast and fungi. Soluble β-glucan can be detected in the sera of patients suffering deep mycotic infections and febrile episodes due to fungemia (27). Originally identified as the component of zymosan responsible for

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[3] Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; ECM, extracellular matrix; Fn, fibronectin; VLA, very late Ag protein; MEM, minimal essential medium; dpm, disintegrations per minute.

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macrophage activation (28), β-glucans have been shown to stimulate hemopoietic immune effector cells in both in vivo and in vitro models (29, 30). However, the mechanism by which leukocytes recognize and respond to soluble β-glucan has not been determined. Based upon evidence that interaction of soluble β-glucan with Mac-1 results in altered effector cell behavior, the possibility that the regulatory effect of β-glucan extends to additional integrin-mediated functions was examined. The current investigation tests the hypothesis that, in the context of extracellular matrix, β-glucan alters the migratory behavior of neutrophils.

Materials and Methods

Isolation of neutrophils

Human neutrophils were isolated from the peripheral venous blood of healthy volunteers in heparinized Vacutainer tubes (Becton Dickinson, Lincoln Park, NJ). Granulocytes were prepared by gradient centrifugation on Ficoll-Hypaque (Sigma, St. Louis, IL), followed by erythrocyte sedimentation with 3% dextran. The leukocyte-rich supernatant then underwent hypotonic lysis of contaminating erythrocytes. Cells were resuspended in ice cold minimal essential medium (MEM) (Life Technologies, Grand Island, NY) and visually enumerated. PMN purity and viability were consistently greater than 95%.

Polysaccharides

Dextran (MW 460,500) and mannan were purchased from Sigma. PGG-Glucan and ^{3}H-labeled PGG-Glucan were provided by Alpha-Beta Technology, Worcester, MA. In this report, β-glucan refers to PGG-Glucan.

Commercially available mannan is known to contain a small percentage of contaminating β-glucan, which was removed as described (31) using Con A Sepharose (Pharmacia Biotech, Uppsala, Sweden). A 5-ml column was equilibrated in 0.10 M sodium acetate buffer with addition of 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ (pH 5.2) (Con A buffer). Mannan reconstituted to 4 mg/ml in sterile, endotoxin-free distilled water was applied at 25°C to the column; contaminating β-glucan passed through and was discarded. Bound mannan was eluted with a 4% solution of α-methyl-D-mannopyranoside (Aldrich Chemical, Milwaukee, WI) in Con A buffer. Flow-through carbohydrate was collected, dialyzed against PBS, and lyophilized. Carbohydrate concentration of the lyophilized product was determined via phenol-sulfuric acid assay (32).

All reagent used contained less than 0.1 pg/ml endotoxin as determined by *Limulus amoeba* screening (BioWhittaker, Walkersville, MD). When necessary, endotoxin removal was achieved using immobilized Polymyxin B (Affinity Pak Detoxi-Gel, Pierce, Rockford, IL) followed by repeat *Limulus* testing.

Monoclonal Abs

The following mAbs were employed in adhesion and in situ migration assays, each at a final concentration of 10 μg/ml: Anti-VLA-2, -3, -4, -5, and -6 (Upstate Biotechnology, Lake Placid, NY); 3S3 (anti-β_1) (Chemicon International, Temecula, CA); TS 1/18 (anti-β_2, CD18 specific), mAb 44 (anti-Mac-1), anti-LFA-1, and anti-p150/p95 (all purchased from Endogen, Woburn, MA; mAb ICRF44 (anti-Mac-1, nonblocking) (PharMingen, San Diego, CA); mAb VIM12 (anti-Mac-1, activating) (Caltag Laboratories, Burlingame, CA); and mAb 24 (anti-$\alpha_{L,M,X}$, activating) (generously provided by Dr. Nancy Hogg, Imperial Cancer Research Fund, London, U.K.).

Slide preparation

Two-well chambered slides (Lab-Tek Permanox Chambered Slides, Fisher Scientific, Fair Lawn, NJ) were pretreated with purified, endotoxin-free human Fn (Collaborative, Bedford, MA) reconstituted in sterile distilled water to a concentration of 6 μg/ml. Two milliliters was placed in each well, and the slides were incubated at 37°C in 7% CO_2 for 30 min, after which 100 μl soluble β-glucan (1 mg/ml, Alpha-Beta Technology) was added for an additional 30 min. Before use, wells were washed with PBS and allowed to air dry. In some experiments, Fn was substituted with fibrinogen, vitronectin, collagen type IV, polyD-lysine (all from Collaborative Biomedical Products), or heparin sulfate (Sigma) at equivalent concentrations. In experiments regarding PMN adhesion, 96-well plates (Falcon Labware, Becton Dickinson) were treated with reagents at the above concentrations but filled to a volume of 200 μl.

To determine the binding efficiency of β-glucan to ECM components, β-glucan was oxidized with a 20-fold molar excess of sodium periodate for 72 h in the dark, dialyzed against water and reductively radiolabeled with 100 mCi of NaB^3H_4 (New England Nuclear, Boston, MA). The [^{3}H]β-glucan was purified by dialysis and ultrafiltration and achieved a sp. act. of 6.8×10^6 dpm/μg hexose. Approximately 2×10^5 dpm were added to 100 μg cold β-glucan for use as an indicator of binding efficiency to matrix components coated onto chambered slides as described above. Following incubation for 30 min at 37°C, slides were rinsed to remove unbound β-glucan. Remaining β-glucan was eluted using 1% SDS and [^{3}H]β-glucan quantitated by liquid scintillation counting.

Migration assays

Molten agarose (Seakem GTG, FMC Bioproducts, Rockland, ME) was prepared by a modification of the method described (33). Briefly, 1% agarose was boiled in sterile, endotoxin-free sterile saline, followed by 1:1 dilution with MEM to achieve a final agarose concentration of 0.5% that was then distributed into the precoated chambered slides. Using a plastic template and beveled punch, three 2-mm wells were created, each separated by a distance of 2 mm. The agarose plugs were removed with gentle aspiration. The central well received 20 μl of prepared cells in MEM at a concentration of 2×10^7 cells/ml. Ten microliters of 1.0-nM fMLP (Sigma) was placed in the upper left well and 10 μl of PBS in the lower right well (negative control). The slides were incubated for 4 h at 37°C with 7% CO_2 and then formalin-fixed for 10 min. After removal of the agarose, the cells were stained with 2% crystal violet for 5 min. Migration was assessed via Microprojector magnification (Bausch & Lomb, Rochester, NY). A metric ruler was placed over the projected image, and the distance from the outer edge of the center well to the leading edge of the migratory cells was measured. One millimeter magnified represents 0.03 mm actual distance migrated. Both random (chemokinesis; distance toward PBS) and directed (chemotaxis; distance toward fMLP) migration were measured in millimeters. Directed migration = (migration toward chemoattractant) − (migration toward PBS).

Some experiments employed HBSS without Ca^{2+}, Mg^{2+}, or Mn^{2+} (Life Technologies). To evaluate the contributions of individual metals, divalent cations were selectively added to the agarose (in HBSS) at concentrations of 1 mM Ca^{2+}, 2 mM Mg^{2+}, and 0.1 mM Mn^{2+}.

When indicated, PMN were preincubated for 20 min at 37°C with the following peptides purchased from Peninsula Laboratories (Belmont, CA): Arg-Gly-Asp (RGD), RGDS, and RGDC, each at 0.25 mg/ml.

Adhesion assays

Cells (5×10^5) were applied to precoated 96-well plates as described above. Pretreatment of plastic slides or 96-well plates with either native or heat-denatured BSA to block nonspecific binding was without effect in the migration and adhesion assays (data not shown). Nonadherent cells were removed by sealing the plates with an adhesive film and centrifugation at $800 \times g$ for 5 min in an inverted position. Adherent cells were stained with 75 μl of 2% crystal violet for 5 min. The stain was aspirated, the plates were rinsed in PBS, and 100 μl of 10% acetic acid was added to each well. OD was then measured at 550 nm via a Bio-Tek (Winooski, VT) plate reader equipped with DeltaSoft (BioMetallics, Princeton, NJ). The number of cells used was within the linear range of the assay. In some experiments PMN were pretreated with saturating concentrations of mAbs for 15 min at 21°C to identify the adhesive contributions of individual integrin receptors.

FACS analysis

Samples of 2×10^6 isolated PMNs were blocked in ice cold RPMI 1640 containing 10% FBS, 1% normal goat serum, and 0.1% sodium azide (binding buffer) for 30 min on ice. Cells were stained with 10 μg purified mAb for 1 h on ice in a total volume of 100 μl binding buffer. Cells were then washed twice and incubated with 30 μg PE-labeled goat F(ab′)$_2$ anti-mouse IgG (Sigma) for 30 min on ice. Cells were washed twice and resuspended in 1% paraformaldehyde in PBS. Analysis was performed on a FACScan (Becton Dickinson) using Becton Dickinson Lysis II Software.

Statistical analysis of results

When appropriate, data were analyzed using ANOVA with post hoc analysis via the Scheffe F test or the unpaired Student *t* test, as indicated.

Results

β-glucan in the context of ECM converts neutrophil chemokinesis to chemotaxis

Initial experiments were designed to determine the effect of β-glucan on the integrin-mediated chemotactic response of neutrophils. To this end, Fn ± β-glucan was applied to plastic slides. The

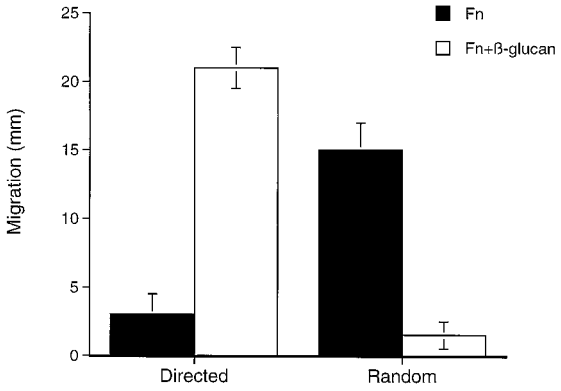

FIGURE 1. PMN migration on Fn and Fn + β-glucan. PMN were seeded into the central well of an agarose slide pretreated with Fn or Fn-β-glucan. After 4 h incubation, migration was measured toward saline (random migration; chemokinesis) or 1.0 nM fMLP (directed migration; chemotaxis). Migration values were as follows: chemokinesis on Fn, 15 ± 3 mm; chemotaxis on Fn, 3 ± 2 mm; chemokinesis on Fn + β-glucan, 3 ± 1 mm; chemotaxis on Fn + β-glucan, 22 ± 3 mm. Data shown represent mean ± SEM of triplicate determinations representative of six separate experiments.

results showed a conversion of chemokinesis (random migration) to chemotaxis (directed migration) in response to 1.0 nM fMLP occurring at a coating concentration of 100 μg β-glucan per 6 μg Fn per well (Fig. 1). This concentration of β-glucan was determined to be optimal for promotion of chemotaxis on Fn (data not shown). The presence of β-glucan was associated with an approximate 7-fold increase in the chemotactic response, and a 5-fold decrease in the chemokinetic response. Binding studies employing ^{3}H-labeled β-glucan determined that 0.1–0.4 μg of bound β-glucan in the context of Fn was sufficient to result in conversion of chemokinesis to chemotaxis (data not shown).

Comparison to a panel of polysaccharides demonstrated yeast-derived β-glucan to be most effective in terms of impact on neutrophil chemotaxis (Fig. 2). Supplementation of Fn with dextran or mannan resulted in migratory responses not significantly different from control.

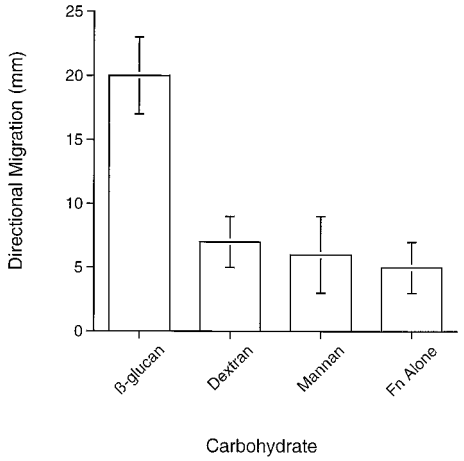

FIGURE 2. A shift from random to directed migration on Fn is stimulated by β-glucan and not by other structurally related polysaccharides ($p < 0.05$). All polysaccharides were applied at a coating concentration of 100 μg/ml. Data represent mean ± SD of triplicate determinations representative of four separate experiments.

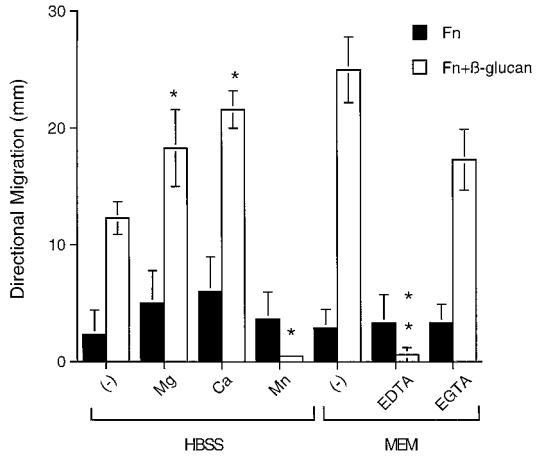

FIGURE 3. Effect of divalent cations on PMN migration. Agarose was prepared using HBSS without divalent cations; HBSS supplemented with 2 mM Mg^{2+}, 1 mM Ca^{2+}, or 0.1 mM Mn^{2+}; or MEM supplemented with 1 mM EDTA or 1 mM EGTA. PMN were resuspended in the buffer used to prepare the agarose. Significant differences were determined on Fn + β-glucan for HBSS without divalent cations vs HBSS + Mg^{2+}, HBSS + Mn^{2+}, and HBSS + Ca^{2+} (*, $p < 0.05$) and MEM vs MEM + EDTA (**, $p < 0.01$). Data shown represent mean ± SD of triplicate determinations representative of three separate experiments.

PMN migration on β-glucan-treated Fn is cation dependent

To assess the potential involvement of integrins in the conversion of chemokinesis to chemotaxis, experiments were conducted to evaluate the impact of divalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) in the agarose media. Chemotaxis on Fn + β-glucan was maximal in the presence of MEM, calcium, or magnesium (Fig. 3). In contrast, the presence of 0.1 mM Mn^{2+} resulted in complete inhibition of chemotaxis on Fn + β-glucan. EDTA profoundly inhibited chemotaxis on Fn + β-glucan while EGTA did not, suggesting that β-glucan promotes chemotaxis by a mechanism that requires the presence of either extracellular calcium or magnesium. In contrast, chemotaxis on Fn occurred independently of specific cations.

β-glucan in the context of alternative ECM proteins failed to influence PMN migration

To determine whether the effect observed in the presence of β-glucan was related to a Fn-specific interaction, slides were coated with alternative matrix proteins, slides were treated with β-glucan, and the migration assay was performed. Neither plastic alone (data not shown) nor poly-D-lysine was an effective substrate for β-glucan-mediated effects. Substitution of Fn by type IV collagen, fibrinogen, heparin sulfate, or vitronectin abrogated the prochemotactic influence of β-glucan (Fig. 4). These matrix components were selected for study since they are normally found in the endothelial basement membrane or in the ECM in tissues and are therefore likely to be encountered by inflammatory neutrophils following extravasation. Interestingly, chemotaxis on type IV collagen was inhibited by addition of β-glucan. Binding studies using ^{3}H-labeled β-glucan failed to detect quantitative differences in the capacities of the aforementioned matrix components to bind β-glucan, indicating that preferential binding does not account for the effect on migration noted to occur specifically on fibronectin (data not shown).

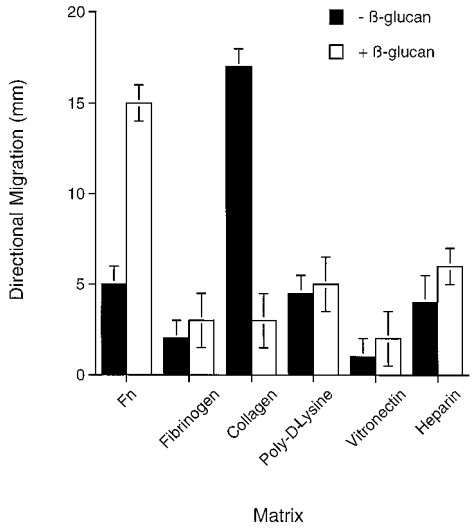

FIGURE 4. PMN chemotaxis on alternative matrix proteins ± β-glucan. Slides were pretreated with Fn, fibrinogen, type IV collagen, vitronectin, or heparin sulfate as described in *Materials and Methods*. Relative to other matrix proteins, the ability of β-glucan to promote PMN chemotaxis is specific to presentation within the context of Fn ($p < 0.05$). Addition of β-glucan to type IV collagen resulted in inhibition of chemotaxis ($p < 0.05$). Data shown represent mean ± SD of triplicate determinations representative of three separate experiments.

RGD oligopeptides inhibit chemotaxis on Fn but not on Fn treated with β-glucan

To determine whether the prochemotactic effect of β-glucan was mediated via an RGD recognition sequence, PMN were preincubated with RGD-containing peptides and applied to the migration assay. On Fn, RGD and RGDS completely inhibited chemotaxis (Fig. 5), which is consistent with a role for VLA-5 in mediating migration on Fn. In contrast, chemotaxis on Fn + β-glucan was diminished by 30% following pretreatment of PMN with RGD and RGDS. The RGDC peptide sequence failed to alter migration on either substrate. Therefore, the prochemotactic influence of β-glucan-supplemented Fn appears to be predominantly mediated

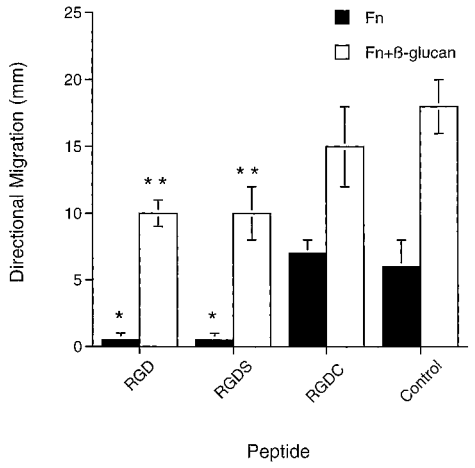

FIGURE 5. Inhibition of PMN chemotaxis by RGD peptides. PMN were pretreated with 0.25 mg/ml RGD, RGDS, or RGDC as described in *Materials and Methods* and applied to the migration assay. RGD and RGDS achieved complete inhibition of chemotaxis on Fn (*, $p < 0.01$) but only partial inhibition (30%) on Fn + β-glucan (**, $p < 0.05$). Data represent mean ± SD of triplicate determinations representative of three separate experiments.

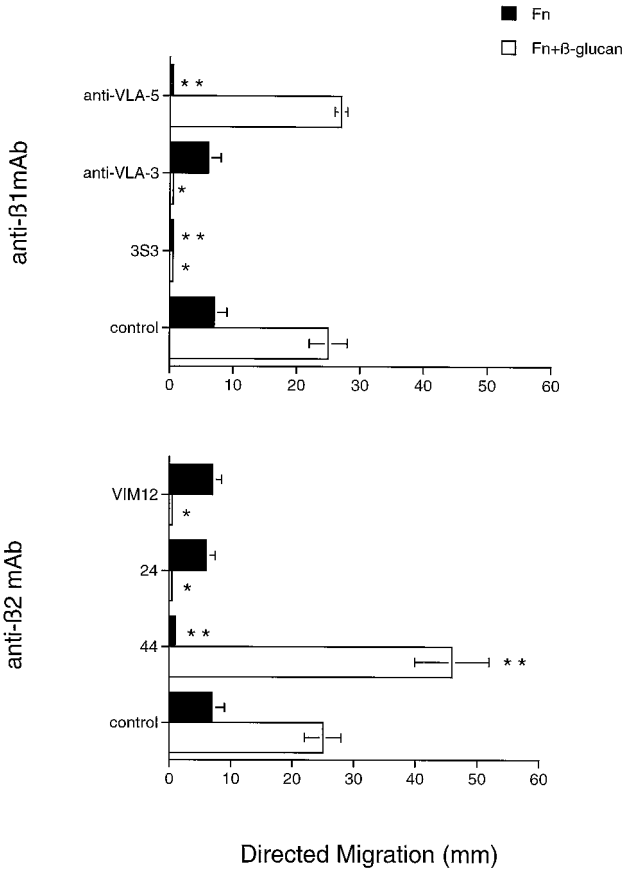

FIGURE 6. Inhibition of PMN chemotaxis by mAbs specific for various β_1 and β_2 integrins. PMN migrated on Fn ± β-glucan under conditions of mAb incorporation in the agarose (10 μg/ml). Included were the following: 3S3 (anti-β_1); anti-VLA-3 and anti-VLA-5 (directed at distinct α subunits associated with the β_1 subunit); mAbs 44, 24, and VIM12 (all directed at the αM subunit, or Mac-1). Isotype-matched IgG served as the control mAb and was without effect relative to untreated (not shown). Data represent mean ± SD of triplicate determinations and are representative of three separate experiments. *, $p < 0.01$; **, $p < 0.02$.

through a non-RGD-dependent mechanism. Taken together, the effect on directed migration on Fn + β-glucan seen by addition of Mn^{2+}, as well as the RGD independent nature of this migration, is exactly consistent with the distinctive pattern of regulation of the β_1 integrin VLA-3, as reported by Hemler and coworkers (Refs. 34 and 35; see *Discussion*).

mAb inhibition studies

To identify the adhesion molecules involved in conversion of chemokinesis to chemotaxis on Fn + β-glucan, PMN migration was assessed under conditions of mAb incorporation in the agarose media (Fig. 6). The involvement of members of the β_1 integrin family was elucidated using mAb 3S3 directed at the common β_1 subunit along with Abs that react specifically with VLA-1 to -6. The anti-CD11b mAb VIM12 has been shown to block binding of β-glucan (25) and was therefore tested in chemotaxis assays along with the activation/reporter Ab mAb 24 and mAb 44, which bind to the I-region of CD11b. Consistent with previous reports (36), preliminary experiments revealed presentation of Abs via the agarose to be the most effective means of inhibiting migration (data not shown). On Fn, chemotaxis was inhibited by the anti-β_1 mAb 3S3 (96%), anti-VLA-5 (85%), and the Mac-1 function-blocking mAb 44 (85%). On Fn + β-glucan, chemotaxis was completely

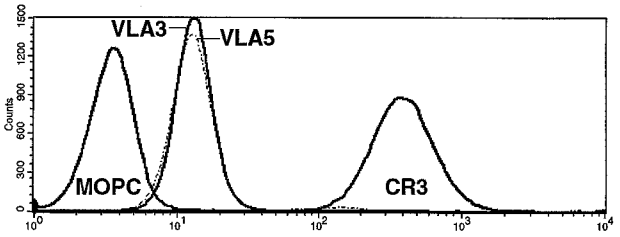

FIGURE 7. Immunofluorescent staining of integrins expressed on PMNs immediately following isolation. Neutrophils were stained with MOPC (isotype control), anti-VLA-3 (Upstate Biotechnology; solid line), anti-VLA-5 (Upstate Biotechnology; hatched line), or anti-CR3 (PharMingen) as indicated and analyzed by flow cytometry as described in *Materials and Methods*. Log scale of fluorescent intensity is shown on the *x*-axis. Data are from a representative of four independent experiments.

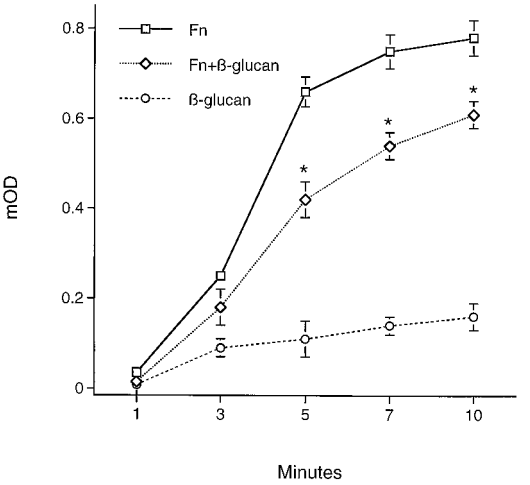

FIGURE 8. PMN adhesion to Fn, Fn + β-glucan, and β-glucan. Microtiter plates were pretreated with Fn (6 μg/ml), β-glucan (100 μg/ml), or Fn + β-glucan as described in *Materials and Methods*. PMN were permitted to adhere to the various surfaces for the time indicated followed by removal of nonadherent cells via inverted centrifugation. *, Adhesion to Fn + β-glucan is different from other groups ($p < 0.05$). Data shown represent mean ± SD of quadruplicate determinations and are representative of three separate experiments.

inhibited by 3S3, anti-VLA-3, and the Mac-1-activating mAbs VIM12 and -24. In marked contrast to its inhibitory effect on Fn, mAb 44 promoted chemotaxis on Fn + β-glucan 50–100% ($p < 0.02$). mAb directed at the common β_2 subunit (TS1/18) failed to inhibit chemotaxis on Fn + β-glucan and resulted in minimal inhibition of chemotaxis on Fn alone (14%) (data not shown). Abs directed at the β_1 integrins VLA-2, -4, and -6 failed to affect migration on Fn or Fn + β-glucan, as did the nonblocking mAb ICRF44 (data not shown). Together, these data suggest that both VLA-5 and Mac-1 mediate chemotaxis on Fn, while chemotaxis in the presence of β-glucan is mediated predominantly by VLA-3.

Cell migration mediated by VLA-3 on Fn has been previously reported in motile melanocytes (37). However, since VLA-3 has not previously been shown to direct the migration of human neutrophils, Fig. 7 is included to demonstrate the levels of expression of VLA-3, VLA-5, and CR3. It is noteworthy that virtually 100% of neutrophils assayed immediately following isolation contained immunodetectable levels of all three cell surface molecules such that it is unlikely that commitment to random or directed motility in the assays performed is due to altered expression of the controlling integrins. Pretreatment of PMNs with either 10^{-9} or 10^{-10} M fMLP for 60 min before immunostaining did not alter the percentage of cells coexpressing these molecules (data not shown).

PMN adhesion is diminished on Fn supplemented with β-glucan compared with Fn alone

Optimal speed of cell migration has been shown to occur at an intermediate ratio of cell-substratum adhesiveness such that the cell can form new attachments to matrix at the leading edge while breaking attachments at the rear of the cell (38). The effect of β-glucan supplementation of fibronectin on neutrophil adhesion was tested in a short term adhesion assay that has been shown to represent the dynamic interaction of attachment formation and disruption as it occurs in a rapidly migrating cell. Results of PMN adhesion to matrix substrates is shown in Fig. 8. Relative to Fn, PMN adhesion to Fn + β-glucan was diminished by 29% at 5 min and by 19% at 10 min. Cell adhesion to β-glucan alone was negligible. These findings are consistent with the interpretation that one aspect of the ability of β-glucan to promote directional migration is related to its ability to quantitatively modify neutrophil adhesiveness and thereby facilitate migration.

To determine whether the integrins found to be relevant to motility of neutrophils on Fn + β-glucan were also involved in modifying strength of adhesion, PMNs were pretreated with saturating concentrations of inhibiting mAb before application to Fn ± β-glucan-treated plates. Differing contributions of β_1 and β_2 integrins were observed with regard to PMN adhesion (Table I). On both Fn and Fn + β-glucan, adhesion was maximally and similarly inhibited under conditions of Mac-1 blockade with mAb 44 (63% and 60%, respectively), yet this Ab exhibited opposing effects on directional migration, causing inhibition on Fn and promotion on Fn + β-glucan. VIM12, which binds to an epitope on the Mac-1 molecule in the vicinity of the lectin site (25), resulted in substantial inhibition of PMN adhesion to Fn (50%) but, unlike mAb 44, did not effect migration on Fn. Interestingly, VIM12 resulted in enhanced adhesion of PMNs to Fn + β-glucan (20% increase), quantitatively replenishing the reduction in adhesion by β-glucan as seen in Fig. 8. Were this reduction in adhesion by β-glucan solely responsible for increased directional migration, then an anticipated outcome of the increased adherence of VIM12 to Fn + β-glucan would be migration on β-glucan-supplemented Fn equivalent to that on Fn alone rather than the observed absence of migration (Fig. 6). Finally, although TS1/18 quantitatively reduced adhesion on Fn to the same extent as β-glucan, it failed to promote chemotaxis on Fn and was without effect on Fn + β-glucan. Neither TS1/18 nor anti-VLA-5 appreciably affected PMN adhesion to Fn + β-glucan. No alteration in adhesion was observed with Abs directed at alternative β_2 integrins (LFA-1 and p150/95) (data not shown). Taken together, four different Abs were found to reduce adhesion to Fn over a range of extents; however, none resulted in promotion of directed migration. Since a reduction of adhesion to

Table I. *Effects of β_1 and β_2 MAb on PMN Adhesion*

mAb	Specificity	% Change in Adhesion[a]	
		Fn	Fn + β-glucan
44	CD11b	-63 ± 4^b	-60 ± 6^b
VIM12	CD11b	-50 ± 4^b	$+20 \pm 3^b$
TS1/18	CD18	-20 ± 4^b	-2 ± 2
VLA-5	CD49e	-35 ± 6^b	-5 ± 3
IgG	Fcγ	100	100

[a] Mean ± SD % change in cell adhesion to Fn ± β-glucan at 5 min (three experiments consisting of four determinations for each preparation).
[b] $p < 0.05$ compared with IgG control.

Fn by these Abs did not correlate with an alteration in migration, the prochemotactic effect of β-glucan may be due only in part, but not entirely, to its effect on adhesion.

Discussion

This investigation shows that β-glucan presented in the context of fibronectin regulates integrin-mediated neutrophil migration. Observations reported herein support the conclusion that β-glucan optimizes chemotaxis of neutrophils in response to fMLP through differential regulation of β_1 and β_2 integrins. Consistent with the essential requirement of integrins for extracellular divalent cations, availability of Ca^{2+} or Mg^{2+} was found to be integral to the prochemotactic activity of β-glucan. Migration on Fn appears to be mediated by the joint contributions of Mac-1 and VLA-5, whereas, in the presence of β-glucan, migration is mediated predominantly via VLA-3.

To define the nature of the receptors that mediate chemotaxis in the presence of β-glucan, studies were conducted to evaluate the role of divalent cations (Fig. 3). Cation availability had a significant impact on chemotaxis occurring on Fn + β-glucan. In the presence of MEM, Mg^{2+}, or Ca^{2+}, chemotaxis was optimal. Complete suppression of chemotaxis on Fn + β-glucan was observed in the presence of EDTA but not EGTA, consistent with a process that is operational in the presence of extracellular Mg^{2+} or Ca^{2+}. Consistent with the reported, unique cation sensitivity of VLA-3 (34, 35), 0.1 mM Mn^{2+} effected profound inhibition of chemotaxis on Fn + β-glucan, probably through induction of a high affinity ligand-binding state that obviates migration (see below). More recently, further evidence was obtained for the role of VLA-3 in mediating migration on Fn + β-glucan. CD151, a member of the transmembrane-4 superfamily that exists in a highly stoichiometric association with VLA-3, appears to cooperate with VLA-3 in regulation of PMN motility on Fn in the presence of β-glucan (39).

VLA-3 ($\alpha_3\beta_1$) appears to be unique among the β_1 integrins in several ways. This receptor mediates weakly adhesive interactions to a variety of ligands, including collagen types I and IV, Fn, and laminin (34). VLA-3 recognizes the RGD sequence on Fn, but operates independently of RGD when mediating adhesion to collagen and laminin. The variable RGD dependence of VLA-3 is consistent with the findings reported in the current investigation, which support the involvement of additional non-RGD-dependent receptors (Mac-1). It is further distinguished by ligand-specific cation sensitivities. Of note, in the presence of Mn^{2+}, adherence of VLA-3-transfected K562 cells to Fn is increased 30-fold. The heightened importance of VLA-3 integrins in mediating migration on Fn + β-glucan offers an explanation for the profound inhibition of chemotaxis observed in the presence of Mn^{2+}. Through induction of a high affinity-binding state for Fn, Mn^{2+} served to augment adhesion and diminish migration.

A specificity for the matrix protein Fn was demonstrated by migration experiments in which alternative protein components of the ECM were treated with β-glucan. Substitution with ligands recognized by VLA-6 (laminin) (data not shown), VLA-1 and -2 (collagen), Mac-1 (fibrinogen), and $\alpha_v\beta_3$ (vitronectin) failed to reproduce the enhanced chemotactic response observed on Fn. Treatment of type IV collagen with β-glucan actually resulted in an 83% reduction in directed migration, indicating that potentiation of chemotaxis by β-glucan is integrin specific, since neither Mac-1 nor VLA-5 recognizes type IV collagen as an adhesive ligand. In contrast, Fn permits dual β_1/β_2 recognition, which appears to be essential to the prochemotactic activity of β-glucan.

Discreet roles for individual subtypes of integrin receptors were demonstrated through mAb experiments (Fig. 6). Promotion of chemotaxis by β-glucan was inhibitable by two distinct mechanisms, reflecting a complex mechanism for cell recognition of Fn as a migratory substrate. The prochemotactic effect of β-glucan could be overcome by a blocking Ab directed against VLA-3, indicating the pivotal role of this receptor in manifesting the effects of β-glucan. Interaction of VIM12 with Mac-1 also resulted in inhibition of the prochemotactic effect of β-glucan. The ability of VIM12 to inhibit the activity of β-glucan may be related to the proximity of the VIM12 epitope to the Mac-1 lectin site (40), the binding of which is inhibitable by certain saccharides, including β-glucan and *N*-acetyl-D-glucosamine (25). Chemotaxis on Fn + β-glucan was also inhibited by mAb 24, a unique activation reporter that maintains Mac-1 in an activated, ligand-bound conformation, thus preventing deadhesion (41). Interestingly, Mac-1 blockade with mAb 44, which binds to the ligand-binding I domain, served to potentiate the prochemotactic effect of β-glucan, supporting the hypothesis that β-glucan inhibits Mac-1-mediated adhesion. In contrast, mAb 44 significantly inhibited chemotaxis on Fn alone (85%). The extent of chemotactic inhibition observed in the presence of mAb 44 was equivalent to that seen with anti-VLA-5, which blocks the classic RGD-dependent Fn receptor. These findings support the hypothesis that PMN migration on Fn results from the joint contributions of Mac-1 and VLA-5, whereas, in the presence of β-glucan, VLA-3 is the dominant receptor.

Taken together, these findings suggest collaboration between Mac-1 and VLA-5 as a means of regulating integrin-mediated migrational responses. Cross-talk between integrin subfamilies has been reported (42, 43) and appears to be one way in which adhesive interactions acquire specificity. Activation of individual receptors may lead to relatively nonspecific adhesive interactions, but local communication between activated receptors of different subtypes results in cooperative specificity (44).

Insight into the mechanism by which β-glucan enhances chemotaxis was provided through assays that assessed PMN adhesion to various matrix substrates (Fig. 8). PMN were observed to readily adhere to both plastic (data not shown) and Fn. Relative to Fn alone, adhesion of PMN to Fn in the presence of β-glucan was diminished by 19–29% throughout the 10-min course of the experiment. Extension of the assay to 20 min revealed a slow but progressive equilibration between the two matrices (data not shown). When adhesion was evaluated under conditions of mAb blockade, a significant role for Mac-1 in mediating adhesion to Fn and Fn + β-glucan was demonstrated (Table I). PMN adhesion on Fn was significantly diminished following pretreatment of PMNs with mAbs 44 (63%) and VIM12 (50%). It is noteworthy that these Abs, as well as mAbs TS1/18 and anti-VLA-5, all demonstrated a reduction in adhesion to Fn over a range of extents, yet none manifested an increase in directed migration when included in chemotaxis assays. In contrast to its inhibitory effect on PMN adhesion to Fn, VIM12 actually promoted adhesion to Fn + β-glucan, suggesting that interaction of β-glucan with Mac-1 does not preclude additional interactions, some of which may further regulate chemotaxis.

The adhesion data alone, however, may not entirely explain the ability of β-glucan to enhance chemotaxis, and serve to underscore a complex relationship between adhesion and migration. Specifically, mAb 44 (anti-Mac-1), which blocked adhesion on both Fn and Fn + β-glucan, had contrasting effects with regard to migration; chemotaxis on Fn was inhibited, while that occurring on Fn + β-glucan was promoted. This again opens the possibility of additional effects of β-glucan that serve to influence chemotaxis. Finding that the promotional effect of mAb 44 was not also seen with TS1/18 indicates that interaction with the I-domain of CD11b but not CD18 is responsible for mediating this enhancement.

Findings shown in Table I demonstrate inhibition of PMN adhesion to fibronectin (albeit to varying extents) by Abs directed against Mac-1, which is not noted as a receptor for fibronectin or other proteins found in the ECM. However, other reports have also demonstrated that anti-Mac-1 Abs prevent binding of PMNs to a variety of proteins including fibronectin, vitronectin, collagen, thrombospondin, gelatin, and albumin (45–47). Furthermore, leukocytes obtained from patients with a congenital deficiency in Mac-1 expression also show diminished binding to fibronectin, vitronectin, laminin, and gelatin (45, 48). A definitive mechanism has not been established that reconciles the inability of Mac-1 to demonstrate direct binding to ECM proteins with obviation of Mac-1 function preventing adhesion of cells to these same substrates. However, Davis (47) has shown that denatured proteins such as albumin can serve as effective affinity matrices for recovery of Mac-1, suggesting a model whereby ECM proteins are denatured during leukocyte diapedesis, making them recognizable substrates for β_2 integrins.

To begin to elucidate the intracellular signaling pathways elicited in promotion of chemotaxis in the presence of β-glucan, a preliminary series of migration assays were performed in the presence of kinase inhibitors. These initial experiments indicate that the p38 inhibitor SB203580 may specifically inhibit the ability of β-glucan to enhance directional migration in response to fMLP (data not shown). Of note, fMLP induction of PMN chemotaxis has previously been shown to be dependent upon the ability of p38 MAPK to phosphorylate mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP kinase-2) (49). It is therefore of ongoing interest to determine whether the directional motile response of neutrophils to fMLP occurs via a p38-dependent signal transduction pathway when migrating on Fn + β-glucan.

Evidence for the signaling capacity of β-glucan in human monocytes and neutrophils is provided by recent reports of the activation of an NF-κB-like transcription factor in β-glucan-treated cells. In these studies, unlike LPS treatment, which resulted in formation of the classic p50/p65 heterodimer of NF-κB, β-glucan stimulated the formation of a p65:CCAAT/enhancer binding protein (C/EBP)-β heterodimer (50). Whether this transcription factor plays a role in the anti-microbial or chemotactic effect of β-glucan on human leukocytes is not yet determined.

The biologic relevance of these findings may reside in the in vivo migratory response of neutrophils to sites of fungal infection. Because pathogenic yeast and fungi elaborate soluble β-glucan (27), it is plausible that β-glucan is present in the interstitium surrounding a focus of fungal infection. Thus the migration assay described herein may be representative of conditions that locally influence neutrophil migration in the event of a mycotic infection. The implications of enhanced PMN recruitment under the influence of β-glucan are currently under investigation.

In summary, this report demonstrates a novel mechanism for modulation of integrin-mediated leukocyte migration. Through differential regulation of β_2 and β_1 integrins, chemotaxis may be optimized and chemokinesis suppressed. Currently available data suggest that VLA-3 is the membrane receptor responsible for mediating directional migration on Fn in the presence of β-glucan.

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