

Chemotactic Peptide Modulation of Actin Assembly and Locomotion in Neutrophils

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ABSTRACT To determine the relationship between the state of actin polymerization in neutrophils and the formyl-methionyl-leucyl-phenylalanine (fMLP)-induced changes in the locomotive behavior of neutrophils, the mean rate of locomotion (mROL), the percent G-actin, and the relative F-actin content of neutrophils were determined. The mROL was quantified by analysis of the locomotion of individual cells; the percentage of total actin as G-actin was measured by DNase I inhibition; and the F-actin was determined by fluorescence-activated cell sorter (FACS) analysis of nitrobenzoxadiazol (NBD)-phalloidin-stained neutrophils.

Neutrophils stimulated with fMLP exhibit a change in their mROL that is biphasic and dose dependent. The mROL of neutrophils exposed to 10^{-8} M fMLP, the K_D , is 11.9 ± 2.0 $\mu\text{m}/\text{min}$ (baseline control 6.2 ± 1.0 $\mu\text{m}/\text{min}$). At 10^{-6} M fMLP, the mROL returns to baseline levels. Stimulation of neutrophils with fMLP also induces actin polymerization. Evidence for actin polymerization includes a 26.5% reduction in G-actin and a twofold increase in the amount of NBD-phalloidin staining of cells as determined by FACS analysis. The NBD-phalloidin staining is not due to phagocytosis, is inhibited by phalloidin, requires cell permeabilization, and is saturable at NBD-phalloidin concentrations $>10^{-7}$ M. The fMLP-induced increase in NBD-phalloidin staining occurs rapidly (<2 min), is temperature dependent, and is not due to cell aggregation. Since NBD-phalloidin binds specifically to F-actin, the increase in fluorescent staining of cells likely reflects an increase in the F-actin content of fMLP-stimulated cells. FACS analysis of NBD-phalloidin-stained cells shows that the relative F-actin content of neutrophils stimulated with 10^{-11} – 10^{-8} M fMLP increases twofold and remains increased at concentrations $>10^{-8}$ M fMLP. Therefore, the fMLP-induced increase in F-actin content of neutrophils as determined by FACS analysis of NBD-phalloidin-stained cells coincides with a decrease in G-actin and correlates with increased mROL of neutrophils under some (10^{-11} – 10^{-8} M fMLP) but not all ($>10^{-8}$ M fMLP) conditions of stimulation. Quantification of the F-actin content of nonmuscle cells by FACS analysis of NBD-phalloidin-stained cells may allow rapid assessment of the state of actin polymerization and correlation of that state with the motile behavior of nonmuscle cells.

Actin is a ubiquitous protein in eucaryotic cells. It exists in globular (43,000 dalton) and filamentous forms. The filamentous form (F-actin) interacts with myosin to generate the force necessary for motility in nonmuscle cells. In addition, F-actin, in conjunction with other cytoskeletal proteins, plays a major structural role within nonmuscle cells. Presumably in nonmuscle cells, as in muscle cells, actin interacts with myosin in accord with the "sliding filament hypothesis" to generate the contractile force necessary for motile behavior (1). Force generation and movement of cellular components requires the presence of myosin, ATP, and filamentous actin (2).

In its structural role, actin interacts with a variety of proteins in nonmuscle cells (3, 4). These include bundling proteins like α -actinin which bundle actin filaments into stress fibers (5), and high molecular weight proteins, like spectrin (6) and actin-binding protein of neutrophils (7), which cross-link actin to form two-dimensional (3, 6) or three-dimensional cytoskeletal networks (8–10) within the cytoplasm or in association with the plasma membrane. These cytoskeletal networks determine the shape, the organelle distribution, and the cytoplasmic consistency of cells and, through their assembly and disassembly, are probably responsible for gel-sol transfor-

mations in motile nonmuscle cells (3).

To participate in the generation of force and the formation of a cytoskeleton, actin must be in filamentous form (F-actin). However, the majority of actin in nonmuscle cells exists as either monomers or oligomers bound to accessory proteins (4, 11). Therefore, if the proposed force-generating and structural roles of F-actin are relevant to the motility of nonmuscle cells, then changes in the motile behavior of nonmuscle cells may require a change in the state of actin polymerization.

Actin polymerization is thought to occur during the thrombin-induced shape change of platelets (12, 13), and the capping of concanavalin A receptors on lymphocytes and neutrophils (14). In these instances, investigators have documented a decrease in G-actin using the DNase I inhibition assay (15) and inferred that G-actin polymerized to F-actin. Changes in the amount of F-actin were not directly measured.

Quantification of F-actin is difficult. While increases in "cytoskeleton-associated actin" were noted in thrombin-stimulated platelets and formyl-methionyl-leucyl-phenylalanine (fMLP)¹-stimulated neutrophils by SDS PAGE (10–12), conclusive evidence that actin polymerization, i.e., a decrease in G-actin and an increase in F-actin, is associated with a change in the locomotive behavior of nonmuscle cells is lacking.

The studies reported here examine a quantifiable, motile behavior of human neutrophils, the chemokinetic response to fMLP, and measure the percentage of total actin as G-actin and the relative F-actin content of stimulated and unstimulated cells. The chemokinetic response of neutrophils is the change in rate of locomotion of neutrophils induced by stimulation with fMLP in the absence of a gradient (16). The relative F-actin content of fMLP-stimulated and unstimulated cells was measured using nitrobenzoxadiazol (NBD)-phalloidin staining and a fluorescence-activated cell sorter (FACS). These studies show that (a) the relative F-actin content of neutrophils can be measured by FACS analysis; and (b) formyl-met-leu-phe induces actin to polymerize within neutrophils as evidenced by a twofold increase in relative F-actin content and a 26.5% decrease in G-actin following fMLP stimulation (3). The increase in relative F-actin content of fMLP-stimulated neutrophils correlates with an increase in the rate of locomotion of neutrophils under some (10^{-11} – 10^{-8} M fMLP), but not all (10^{-7} – 10^{-6} M fMLP), conditions of stimulation. These findings suggest that the rate of locomotion of neutrophils is related to the amount of cellular actin in the polymerized state and that quantification of F-actin content by FACS analysis of NBD-phalloidin-stained cells may be a useful tool for assessing changes in the polymerization state of actin in nonmuscle cells.

MATERIALS AND METHODS

Isolation of Neutrophils: Leukocytes were prepared from human peripheral blood in EDTA anticoagulant by dextran 60 (Cutter Laboratories, Inc., Berkeley, CA) sedimentation, contaminating erythrocytes were removed by brief (45 s) hypotonic lysis, and neutrophils were purified on Ficoll-Hypaque (17) yielding 96–97% neutrophils, 2–3% eosinophils, 0–1% mononuclear cells. All experiments were done within 5½-h in vitro age of the cell (18). Cells were suspended in Hanks'/HEPES buffer for locomotion studies and NBD-phalloidin staining.

Quantification of Neutrophil Staining: Neutrophil locomotion was quantified as previously described (19) with the following modifications.

¹ *Abbreviations used in this paper:* FACS, fluorescence-activated cell sorter; fMLP, formyl-methionyl-leucyl-phenylalanine; mROL, mean rate of locomotion; NBD, nitrobenzoxadiazol.

Neutrophils suspended in Hanks'/HEPES buffer (25 mM HEPES, 50 mM phosphate, 150 mM NaCl, 4 mM KCl, 1.0 mM MgCl₂, 1.2 mM CaCl₂, 0.05% delipidated human serum albumin [Sigma Chemical Co., St. Louis, MO], pH 7.15) were introduced cold (4°C) into a Dvorak-Stotler Chamber (Nicholson Precision, Bethesda, MD) and warmed to 37°C with a cycling servo-controlled infrared incubator (Optiquip, Highlands, NY) which was linked by a gold probe to the chamber. Temperature on the lower coverslip (coverslip for cell attachment) was monitored with a telethermometer and ¼-inch banjo probe. (Yellow Springs Instruments Co., Inc., Yellow Springs, OH). After 10 min at 37°C, the cells were videotaped and tracked as previously described (19). Mean rate of locomotion (mROL) for a group of cells is an average of the rate of locomotion of 85–120 individual cells determined by tracking cells on transparencies during two separate 1-min periods (fourth minute and ninth minute) of a 10-min videorecording. The tracings were digitized with a GP-50 sonic digitizer (Scientific Accessories, CT) and analyzed with Cyber 307 computer assistance. fMLP in DMSO (0.1% vol/vol) or DMSO (0.1% vol/vol) alone was added immediately prior to injection of cells into the chamber.

Quantification of Actin by DNase I Inhibition: With some modifications, the DNase assay as described by Blikstad et al. (15) was used to measure the percent G- and total actin pools. For baseline determinations, 3.0 ml of DNA substrate (80 µg/ml calf thymus DNA [Sigma Chemical Co.] dissolved in 0.1 M Tris HCl, 4 mM MgSO₄, 1.8 mM CaCl₂, pH 7.5) was reacted with 20 µl of DNase I (EC 3.1.4.5) type 1, chromatographically purified (Sigma), dissolved initially at 10 µg/ml in 0.125 M Tris HCl, 5 mM MgCl₂, 2 mM CaCl₂, 1 mM NaNa₃, pH 7.5, and then further diluted to 0.1 mg/ml in 20 mM imidazole HCl, 30 mM NaCl, 15% glycerol (20). Standard curves of DNase I inhibitory activity were constructed using rabbit muscle actin prepared by the method of Spudich and Watt (21) and purified by gel filtration on Sephacryl G-200 superfine (Pharmacia Inc., Piscataway, NJ). Since the guanidine HCl inhibits the DNase I reaction, standard curves were constructed with and without the addition of 20 µl of guanidine HCl solution (1.5 M guanidine HCl, 1.0 M NaCH₃CO₂, 1 mM CaCl₂, 1 mM ATP, 20 mM Tris HCl, pH 7.5) to determine total and G-actin, respectively.

To measure actin in cells, we lysed neutrophils in Hanks' buffered salt solution with 0.1 vol of 20 mM MgCl₂, 20 mM EGTA, 2 mM ATP, 5 mM dithiothreitol, 10% Triton X-100. To measure G-actin, we immediately added 20 µl of the lysate to 20 µl of DNase I and then added the combination to 3 ml of DNA substrate. To measure total actin, we incubated 20 µl of the lysate with 20 µl guanidine HCl for 20 min at 4°C, then added the DNase I and substrate. In some experiments, intact neutrophils were first stimulated with various concentrations of fMLP at 37°C for 5 min before lysis. Cell concentrations were adjusted so that the inhibitory activity of 20 µl of the lysate fell between 20 and 70% on both standard curves. All measurements were performed in quadruplicate on a Beckman 35 UV/vis recording spectrophotometer (Beckman Instruments, Inc. Palo Alto, CA) at 260 nm and 30°C.

NBD-Phalloidin Staining of Neutrophils: Neutrophils (0.9 ml of 1.1×10^6 cells/ml) were incubated at 37°C in Hanks'/HEPES buffer with varied concentrations of fMLP in DMSO (0.1% vol/vol) or DMSO (0.1% vol/vol) alone. After 10 min incubation, cells were fixed, permeabilized, and stained in a single step by addition of 0.1 ml of 37% phosphate-buffered formalin containing 1.65×10^{-6} M NBD-phalloidin (Molecular Probes, Junction City, OR) and 100 µg lysophosphatidylcholine. The stain mixture plus cells was incubated for 10 min at 37°C. Cells were centrifuged at 400 g for 5 min at room temperature and resuspended in Hanks'/HEPES buffer before analysis.

For some experiments a two-step stain was used. Cells were incubated at 37°C for 10 min with or without fMLP. In step one, they were fixed and permeabilized for 5 min at 37°C with 0.1 ml of 37% phosphate-buffered formalin containing 100 µg lysophosphatidylcholine; in step two, they were stained for 10 min at 37°C by adding 50 µl of 3.3×10^{-6} M NBD-phalloidin in Hanks'/HEPES buffer. Staining results are similar for cells fixed for 5–20 min at 37°C. Cell preparations for microscopic analysis were made on a cytocentrifuge (Shandon Southern cytocentrifuge [Shandon Southern Instruments Inc., Sewickley, PA], spun at 1,000 rpm for 5 min) and are referred to as cytopins in the text.

FACS Analysis of NBD-Phalloidin-stained Cells: FACS analysis was completed within 1 h of cell resuspension. The results were similar for stained cells that were not resuspended in Hanks'/HEPES buffer and stained cells that were resuspended within 1 h before analysis. For most experiments, cells were filtered through a 50-µm nylon mesh filter to remove large aggregates. Results on filtered and nonfiltered samples were similar. Experiments described in Results that evaluate the role of aggregation in increased NBD-phalloidin staining were done without prior filtration. The cells were analyzed on a Becton-Dickinson model IV fluorescence-activated cell sorter (Becton, Dickinson & Co., Oxnard, CA). Cells were excited with an argon laser at 488 nm and emission was read at 522 nm with a long-pass filter. For most experiments, 50,000 cells were analyzed. Results were stored and displayed as histograms of fluorescence (cell number versus fluorescence channel [0–250]) and light scatter

(cell number versus light scatter channel [0–250]). The histograms were recorded on Polaroid positive prints.

We determined relative F-actin content by (a) measuring the area under the histogram for a particular channel; (b) multiplying that area by the channel number; and (c) summing the multiplied areas obtained for a particular histogram to yield an estimate of total fluorescence. The relative F-actin content is expressed as total fluorescence with [fMLP] (in mol/liter)/total fluorescence with DMSO (0.1% vol/vol).

RESULTS

Response of Neutrophil Locomotion to fMLP

To determine the dose response of the mean rate of neutrophil locomotion to fMLP, we measured the rate of locomotion of 85–120 neutrophils in the presence of DMSO alone (0.1% vol/vol) and in the presence of increasing concentrations of fMLP (10^{-11} – 10^{-6} M). Fig. 1 shows the mROL of neutrophils exposed to various concentrations of fMLP from three separate experiments. The mROL of neutrophils increases with increasing concentrations of fMLP over the range of 10^{-11} – 10^{-8} M. The mROL is maximal at 10^{-8} M fMLP (11.9 ± 2.0 $\mu\text{m}/\text{min}$). This maximum value is a twofold increase in mROL above control values in DMSO (6.2 ± 1.0 $\mu\text{m}/\text{min}$). At fMLP concentrations $>10^{-8}$ M, the mROL of neutrophils declines and returns to control levels at 10^{-6} M fMLP. The biphasic dose-response curve for locomotion of neutrophils is similar to that reported for other formylated oligopeptides (22) and the maximum occurs at 10^{-8} M fMLP, the K_D for fMLP binding to the formylated peptide receptor of neutrophils (23).

Characterization of NBD-Phalloidin Staining of Neutrophils

Initially, a single-step stain for F-actin was used and stained cells were analyzed for fluorescence emission at 522 nm using an FACS. The single-step stain requires a 10-min exposure of neutrophils to 3.7% formalin, 100 $\mu\text{g}/\text{ml}$ lysophosphatidylcholine, and 1.65×10^{-7} M NBD-phalloidin at 37°C. Fig. 2 shows the histograms of fluorescence for control cells and cells exposed to 10^{-9} M fMLP. Fluorescence histograms of neutrophils exposed to 10^{-9} M fMLP consistently fell to the

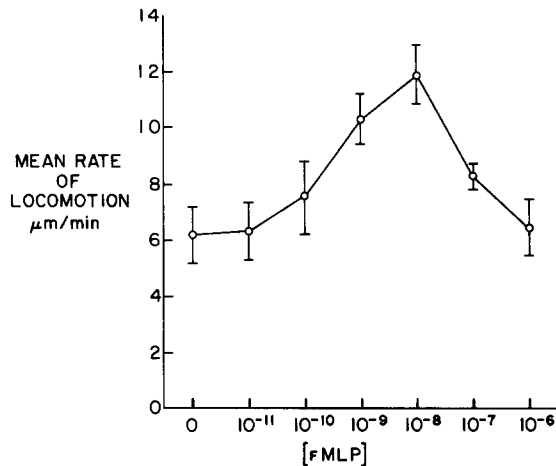


FIGURE 1 Dose response of mean rate of locomotion of neutrophils to fMLP ([fMLP], mol/liter). The mROL of neutrophils in Hanks'/HEPES buffer, pH 7.15, and 0.05% delipidated human serum albumin was determined by single-cell analysis. Points plotted are mROL \pm 1 SD determined from three separate trials with 85–120 neutrophils analyzed at each point in each trial.

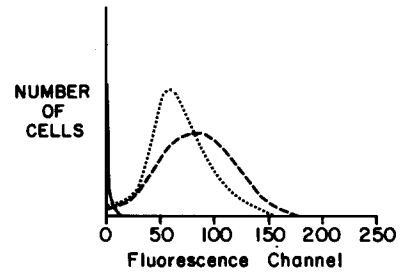


FIGURE 2 Characterization of NBD-phalloidin staining of neutrophils. Data shown are FACS-generated histograms of fluorescence (excitation at 488 nm, emission at 522 nm) for 50,000 cells exposed to 10^{-9} M fMLP (----), 0.1% vol/vol DMSO (.....), 10^{-9} M fMLP plus 1.65×10^{-5} M phalloidin (—), or 10^{-9} M fMLP without addition of the permeabilizer, lysophosphatidylcholine (— · —); the last two exposures resulted in the same histogram.

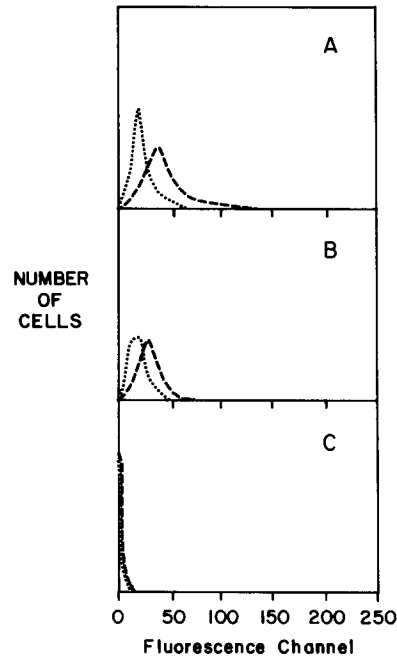


FIGURE 3 Effect of temperature on the NBD-phalloidin staining of neutrophils. Data shown are FACS-generated histograms of fluorescence (excitation at 488 nm, emission at 522 nm) for 50,000 cells exposed to 10^{-9} M fMLP (----) or 0.1% vol/vol DMSO (.....), the carrier of fMLP, at (A) 37°C, (B) 30°C, (C) 4°C. Cells were stained in a single step at 37, 30, and 4°C, respectively.

right of histograms of control cells. The NBD-phalloidin staining is rapid (maximal staining occurs within 2 min), constant for 35 min after stimulation at 37°C, requires permeabilization of the cell (Fig. 2), and is temperature dependent. As shown in Fig. 3, the fMLP-induced increase in NBD-phalloidin staining is decreased at 30°C and at 4°C staining is practically absent. The decrease in NBD-phalloidin staining does not reflect temperature dependence of binding because cells that are initially fixed and permeabilized at 37°C subsequently stain equally well at 4° and 37°C. This result suggests that the number of NBD-phalloidin binding sites is temperature dependent.

The NBD-phalloidin staining of control and fMLP-stimulated neutrophils is specific. Phalloidin, a non-fluorescent congener of NBD-phalloidin, competes with NBD-phalloidin for binding sites on F-actin (24–26). As shown in Fig. 2, NBD-phalloidin staining of control and fMLP-treated neutrophils is abolished by a 100-fold excess of phalloidin. This finding indicates the specificity of NBD-phalloidin staining for a cellular component that also recognizes the nonfluorescent phallotoxin. To determine whether the NBD-phalloidin

staining of neutrophils is saturable, we stained a constant number of neutrophils with increasing concentrations of NBD-phalloidin (1.65×10^{-8} M– 1.65×10^{-6} M) in the presence and absence of a 100-fold excess of phalloidin. These studies show that NBD-phalloidin staining of cells exposed to DMSO, 10^{-8} M fMLP, and 10^{-6} M fMLP is saturated at concentrations $\geq 1.65 \times 10^{-7}$ M (data not shown).

Controls for NBD-Phalloidin Staining

Since neutrophils are avidly phagocytic cells that can aggregate in the presence of fMLP and since phalloidin at equimolar concentrations with G-actin can induce actin polymerization (25), controls are required to exclude phagocytosis, cell aggregation, and phalloidin-induced actin polymerization as the cause of the fMLP-induced increase in NBD-phalloidin staining.

To exclude nonspecific phagocytosis of medium containing NBD-phalloidin as the cause for the fMLP-induced increase in NBD-phalloidin staining, we incubated neutrophils for 10 min at 37°C in the presence of NBD-phalloidin with and without 10^{-9} M fMLP; neutrophils were fixed, stained in the absence of permeabilizer (palmitoyl lysophosphatidylcholine), and analyzed by FACS. As shown in Fig. 2, fluorescent staining was virtually absent from both control and stimulated cells suggesting that staining is not due to phagocytosis of the NBD-phalloidin. Similar results were observed with exposure of the cells to 10^{-8} or 10^{-6} M fMLP.

To exclude fMLP-induced aggregation of neutrophils as the cause for the increased NBD-phalloidin staining observed with fMLP, we performed two experiments. Since neutrophil aggregation should result in a shift in the light scatter histogram, the first experiment compared the FACS-generated histogram of light scatter for control and fMLP-stimulated neutrophils. We found that the histograms are practically identical indicating that under the conditions of these experiments, aggregation of cells is not induced by fMLP (data not shown). Secondly, neutrophils exposed to 10^{-9} M fMLP were stained and sorted into two batches: (1) a less fluorescent batch (cells with less than the mode of fluorescence) and (2) a more fluorescent batch (cells with greater than the mode of fluorescence). This division is indicated by the arrow in Fig. 4A. As illustrated in Fig. 4B, the two batches of fMLP-stimulated cells have similar FACS-generated histograms of light scatter. This result suggests that the broad, right-shifted fluorescence histogram of fMLP-stimulated cells is not due to the presence of aggregates. Finally, microscopic analysis of cytopsin preparations of the two batches of fMLP-stimulated cells and the DMSO-treated cells show that fMLP treatment does not increase the number of neutrophil aggregates or the size of neutrophil aggregates (See Fig. 4C). Similar results are obtained when cytopsin of DMSO and fMLP-treated cells are compared. These results suggest that the fMLP-induced increase in NBD-phalloidin staining is not due to aggregation of cells. Similar results pertain to cells exposed to 10^{-8} or 10^{-6} M fMLP.

Finally, since NBD-phalloidin in equimolar concentrations with G-actin can promote actin polymerization (25), the possibility that the increase in NBD-phalloidin staining is due to phalloidin-induced actin polymerization was considered. To examine this possibility, we divided the single-step staining procedure (10 min at 37°C with 3.7% formalin, 100 μ g/ml lysophosphatidylcholine, 1.65×10^{-7} M NBD-phalloidin) into two steps: Step 1, 5 min at 37°C with 3.7%

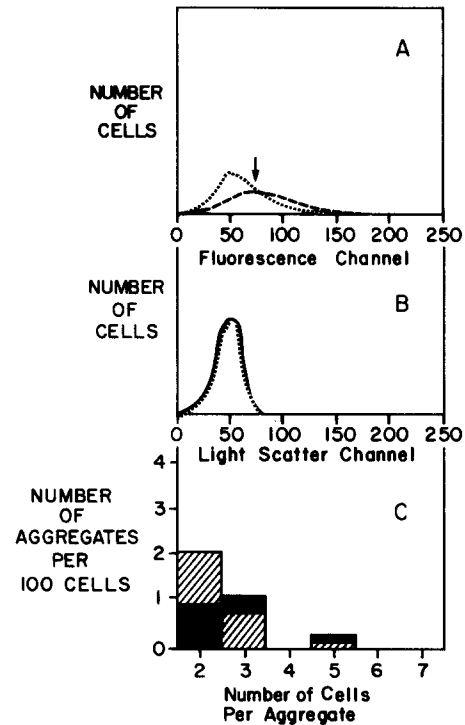


FIGURE 4 Evidence against cell aggregation as the cause of the fMLP-induced increase in NBD-phalloidin staining. The fluorescence histogram of 50,000 neutrophils exposed to 10^{-9} M fMLP (.....) or DMSO 0.1% vol/vol (.....) and stained at 37°C with NBD-phalloidin was determined with the FACS as shown in 4 A on the FACS. Neutrophils were sorted by FACS into a more fluorescent and a less fluorescent batch of cells as defined by the mode of fluorescence (see arrow in 4 A). FACS-generated histograms of light scatter for the more fluorescent (——) and less fluorescent (.....) cells are similar (4 B). Microscopic examination and scoring of 400 consecutive neutrophils in cytopsin preparations from the more fluorescent (▨) and less fluorescent (■) batch of cells were analyzed for number of aggregates per 100 cells and the number of cells per aggregate, as shown in 4 C.

formalin and 100 μ g/ml lysophosphatidylcholine; and Step 2, 10-min exposure at 37°C to 1.65×10^{-7} M NBD-phalloidin. FACS-generated histograms of fluorescence for 50,000 neutrophils stained with either the one- or two-step stain are similar when cells are exposed to DMSO, 10^{-8} M fMLP, or 10^{-6} M fMLP. Similar results are obtained if the fixation and permeabilization period (Step 1) is extended from 5 to 20 min at 37°C. Fixation of cells before exposure to the NBD-phalloidin is presumed to prevent any NBD-phalloidin-induced actin polymerization. These results indicate that in neutrophils, NBD-phalloidin staining is not due to phagocytosis, aggregation, or phalloidin-induced actin polymerization and is inhibited by a nonfluorescent phalloxin, phalloidin. Since NBD-phalloidin binds specifically to purified F-actin *in vitro* (24–26), is localized in intact cells to actin bundles (27), and is localized to the cytoskeleton of Triton-extracted hepatocytes (25), the fluorescence of NBD-phalloidin-stained neutrophils probably reflects the F-actin content of the cell. Furthermore, F-actin content of neutrophils increases upon stimulation with fMLP.

Dose Response of F-Actin Content to fMLP

Neutrophils exposed to increasing concentrations of fMLP (10^{-11} – 10^{-6} M), stained with NBD-phalloidin, and analyzed

for fluorescence by FACS exhibit a progressive increase in relative F-actin content as judged by the mode of fluorescence on the fluorescence histogram (Fig. 5). Integration of the areas under the histograms of fluorescence for control and stimulated cells show that after exposure to 10^{-11} – 10^{-8} M fMLP, neutrophils exhibit a twofold increase in F-actin content relative to control neutrophils (Fig. 6). The F-actin content is constant at fMLP concentrations $>10^{-8}$ M. The data suggest that fMLP-stimulation of neutrophils induces actin polymerization and results in a progressive increase in the F-actin content of cells.

If actin polymerization results from fMLP stimulation of neutrophils, then a decrease in G-actin should occur concurrent with the increase in F-actin. This phenomenon is observed when, as shown in Fig. 7, G-actin is measured by DNAse I inhibition (16). As shown in Fig. 7, the percent of total actin as G-actin decreases from $79.0 \pm 9.9\%$ in control neutrophils to $59.5 \pm 5.9\%$ and $52.5 \pm 5.5\%$ following stim-

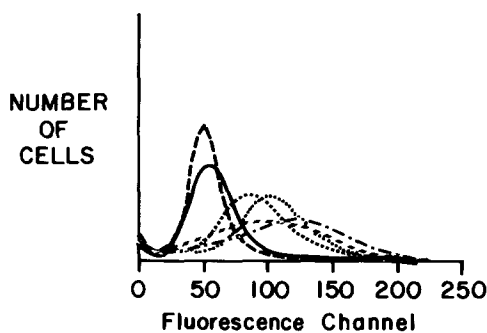


FIGURE 5 Dose response of the NBD-phalloidin staining of neutrophils to fMLP. Neutrophils in suspension were exposed to DMSO (0.1 vol/vol) or 10^{-11} M (-----), 10^{-10} M (—), 10^{-9} M (—●—●), 10^{-8} M (.....), 10^{-7} M (—■—■), or 10^{-6} M (—□—□) fMLP at 37°C for 10 min and stained in a single step. Shown are the FACS-generated histograms of fluorescence for 50,000 cells exposed to the carrier solvent DMSO, or the indicated concentration of fMLP. Similar results are obtained with a two-step staining procedure.

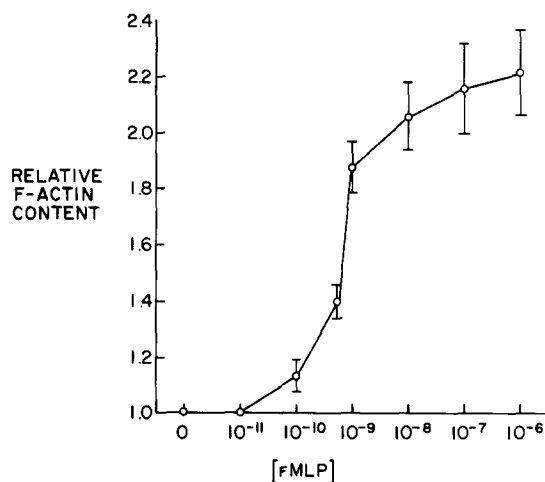


FIGURE 6 Dose response of relative F-actin content of neutrophils exposed to 10^{-11} – 10^{-6} M fMLP. Neutrophils in suspension were exposed to DMSO or the indicated concentration of fMLP (mol/liter) at 37°C for 10 min and stained in a single step. The FACS-generated histograms of fluorescence were analyzed as described in Materials and Methods for the quantity of fluorescence of 50,000 cells relative to the DMSO control.

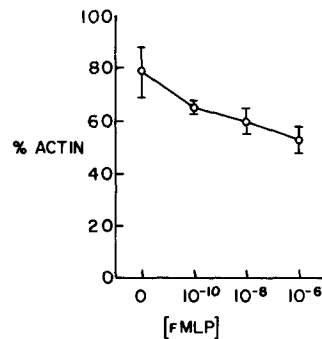


FIGURE 7 Dose response of percent G-actin in neutrophils to fMLP stimulation. Neutrophils were exposed to DMSO (0.1% vol/vol) or the indicated concentration of fMLP (mol/liter) at 37°C for 10 min. The percentage of globular actin was determined with DNase I inhibition by the method of Bliksted and Carlsson (15).

ulation with 10^{-8} and 10^{-6} M fMLP, respectively. These results show that there is an inverse relationship between G-actin measured by DNase I inhibition and F-actin measured by NBD-phalloidin staining; furthermore, the results indicate that G-actin in neutrophils polymerizes after stimulation with the formylated tripeptide, fMLP.

DISCUSSION

The concept that F-actin serves both force-generating and structural roles in nonmuscle cells derives from several lines of cellular and molecular evidence. First, sliding of actin filaments relative to myosin filaments is the basis of skeletal muscle contraction (2). Second, actin-binding proteins, like actin-binding protein of neutrophils, myosin, and actin are found in most nonmuscle cells (1, 3). Thirdly, studies using indirect immunofluorescence labeling show that these proteins co-localize within the cell during motile events such as phagocytosis and locomotion (28–31). Presumably, relocalization of F-actin vis-a-vis actin-binding protein or myosin is essential for the structural rearrangements and force orientation required for phagocytosis and locomotion (28). These findings suggest that F-actin is essential to the motility and structure of nonmuscle cells. However, relatively less is known about how changes in the quantity and state of polymerization of actin relate to changes in cell motility. Since the majority of actin in unstimulated, nonmuscle cells is in globular form and since F-actin is required for force generation and participates in cytoskeletal organization, the production of filamentous actin by polymerization may precede changes in the motile behavior of cells.

Most studies that relate changes in the polymerization state of actin to changes in the motile behavior of nonmuscle cells have focused on the quantification of G-actin as measured by the DNase I inhibition assay. These studies demonstrated decreasing amounts of G-actin associated with thrombin-induced shape change in platelets (12, 13), and concanavalin A stimulation of lymphocytes and neutrophils (14). In these studies, it is presumed that actin polymerization is occurring and that total F-actin is increasing because the level of G-actin measurable by DNase I inhibition decreases.

Changes in the F-actin content of motile cells as they relate to changes in motility are less well characterized. Although qualitative changes in F-actin distribution and increases in the “cytoskeleton-associated actin” of Tritonized cells have been noted following “activation” of motile phenomena in a variety of cells (9, 10), F-actin has not been directly quantified or correlated with alterations in a specific motile behavior of a cell. This paper reports a method for determining the relative content of F-actin in neutrophils by FACS analysis of NBD-phalloidin-stained cells and it correlates the cellular content

of F-actin with the rate of locomotion of neutrophils and the percentage of total actin as G-actin in the cell.

NBD-phalloidin is a fluorescent derivative of phalloidin—a phallotoxin obtained from *Amanita sp* (25). Microscopic studies and quantitative binding studies indicate that NBD-phalloidin binds specifically to F-actin (24, 27). We quantified the relative fluorescence of neutrophils, which were NBD-phalloidin stained and fixed in suspension, with a fluorescence-activated cell sorter and compared the relative content of F-actin in control neutrophils and neutrophils exposed to fMLP. The mROL of neutrophils was determined by computer assisted analysis of time-lapse video recordings of individual cells (19).

Our studies show that exposure of human neutrophils to fMLP results in an increase in NBD-phalloidin staining. The increase in NBD-phalloidin staining occurs rapidly (<2 min), is constant for 35 min, and requires permeabilization of the cell. The increase in staining is not due to nonspecific phagocytosis, cell aggregation, or NBD-phalloidin-induced polymerization of actin. The NBD-phalloidin staining of control and fMLP-stimulated neutrophils most likely represents binding to F-actin because it is specifically displaced by a 100-fold excess of nonfluorescent phalloidin and because a simultaneous decrease in G-actin as determined by DNase I inhibition is observed.

Neutrophils that are exposed to 10^{-11} – 10^{-8} M fMLP exhibit a consistent, progressive increase in F-actin content when compared with control cells exposed to DMSO. Relative F-actin content approaches a maximum, a twofold increase, at 10^{-8} M fMLP. At fMLP concentrations $>10^{-8}$ M, the relative F-actin content of neutrophils remains 2.0–2.2-fold greater than that of control cells. The increase in F-actin is accompanied by a simultaneous decrease in G-actin as determined by DNase I inhibition. The decrease in G-actin by DNase I inhibition parallels the decrease reported by Rao and Varani (14); however, these authors did not directly quantify F-actin. The data indicates that fMLP stimulation of neutrophils induces actin polymerization within the cell.

Changes in mROL were studied in parallel with determinations of relative F-actin content and percentage of total actin as G-actin. The chemokinetic response (change in rate of locomotion of neutrophils in response to fMLP stimulation) was determined from time-lapse videotape recordings. As previously demonstrated using formylated peptides (22), the dose response of mROL is biphasic. The mROL increases at fMLP concentrations of 10^{-11} – 10^{-8} M, is maximal at 10^{-8} M, and rapidly declines to control rates at 10^{-6} M fMLP.

The data indicates that the increase in mROL induced by 10^{-11} – 10^{-8} M fMLP occurs concurrent with an increase in the F-actin content of stimulated neutrophils. The maximum F-actin content and the greatest mROL both occur at the reported K_D for binding of fMLP to neutrophils (23). The findings at 10^{-11} – 10^{-8} M fMLP support the idea that increased availability of filamentous actin is necessary for increased motility of neutrophils. The increase in rate of locomotion may reflect increased force generated through interaction of F-actin with myosin or an alteration of the F-actin within the cytoskeleton which favors gel-to-sol transformations.

Furthermore, the studies show that although availability of increased amounts of F-actin correlates with neutrophil stimulation, the increase in F-actin does not correlate with an increase in cell motility under all conditions of stimulation. Neutrophils exposed to fMLP concentrations $>10^{-8}$ M exhibit

a twofold increase in relative F-actin content; however, the mROL of these cells steadily declines with further increases in fMLP concentration and returns to control levels at 10^{-6} M fMLP. This finding indicates that availability of F-actin to generate contractile force and participate in the cytoskeleton is a determinant of the locomotive behavior of neutrophils under some (10^{-11} – 10^{-8} M fMLP) but not all ($>10^{-8}$ M fMLP) conditions.

The failure of F-actin content and mROL to correlate in cells stimulated with $>10^{-8}$ M fMLP may reflect a difference in the deployment, localization, or form of filamentous actin. For example, at elevated fMLP concentrations the F-actin of neutrophils may serve only a structural role which locks the cytoskeleton into a rigid, gelled state; or the F-actin could be distributed diffusely rather than concentrated at the pseudopodial and uropodial extremes of the cell as shown by Mandel (32). Alternatively, concurrent activation of another cellular function, e.g., adherence, which limits expression of the increased force associated with increased F-actin content may explain the failure of correlation between F-actin content and motile behavior under all conditions of stimulation. For example, neutrophils stimulated in the absence of albumin are maximally adherent cells (33) which move slowly, 1.5 $\mu\text{m}/\text{min}$, and do not exhibit a chemokinetic response to fMLP; however, these cells do exhibit an increase in relative F-actin content identical to that reported for neutrophils in 0.05% horse serum albumin (T. Howard, unpublished results).

Filamentous actin plays an important role in both the motility and the structural organization of nonmuscle cells. Quantification of F-actin is difficult. Our studies suggest that increases in F-actin in nonmuscle cells can be documented directly by FACS analysis of cells stained with NBD-phalloidin. Although we cannot be certain that NBD-phalloidin staining reflects only binding to F-actin, the assumption is reasonable since NBD-phalloidin binds specifically to F-actin in vitro (24, 25), and associates only with the cytoskeleton of nonmuscle cells (25). Although the studies reported here represent semi-quantitative assessments of F-actin, this method with modifications may allow careful quantification of F-actin in large numbers of cells with relative ease. To determine whether relative F-actin content measured by FACS relates to the ROL of individual cells, this method of NBD-phalloidin staining can be modified to pair measurements of the quantity and distribution of NBD-phalloidin staining with the ROL of individual cells attached to a substratum.

Further study is required to elucidate the mechanism for increased NBD-phalloidin staining of fMLP stimulated neutrophils. Regardless of the cause for the increase, the observed increase in staining reflects a major change in the state of actin that is linked to a quantitative change in the locomotive behavior of neutrophils.

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