

Polymorphonuclear Leukocyte Adherence Induces Actin Polymerization by a Transduction Pathway Which Differs from That Used by Chemoattractants

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Abstract. Nitrobenzoxadiazole-phalloidin in combination with quantitative fluorescent microscopy have been used to measure F-actin concentrations in human polymorphonuclear leukocytes (PMN) as they adhere to a plastic surface. Like stimulation with chemoattractants, adherence is associated with a twofold rise in F-actin content. However unlike the rapid rise in F-actin induced by chemoattractants which peaks within 30 s, actin assembly induced by adherence is slower, maximum F-actin values not being observed until 10 min. Furthermore the rise in F-actin induced by adherence is persistent, remaining constant over 60 min while F-actin returns to near basal levels after 20 min exposure to chemoattractant. The combination of ad-

herence (5 min) followed by chemoattractant (FMLP 5×10^{-8} M for 40 s) resulted in an additive rise in F-actin content to greater than threefold over unstimulated values. Unlike chemoattractant induced actin assembly, adherence-associated PMN actin polymerization was not inhibited by pertussis toxin, but was markedly reduced by lowering extracellular Ca^{2+} . Fluorescent micrographs of adherent PMN stained with nitrobenzoxadiazole-phalloidin revealed F-actin in the lamellipodia and in small foci on the adherent surface. These findings suggest that the transduction mechanisms by which adherence induces PMN actin polymerization differ from those used by chemoattractant receptors.

THE cytoplasm of polymorphonuclear leukocytes (PMN)¹ has been estimated to contain 200 μ M actin, \sim 1,000-fold above the critical concentration of purified actin. In unstimulated PMN a high percentage, 60–70%, of the total actin exists as globular actin (G-actin). Chemoattractants rapidly increase the proportion of the total cytoplasmic actin present in a filamentous form (9, 15, 30). Within \sim 30 s, the proportion of F-actin increases from 30 to \sim 60%. Upon continued incubation the level of polymerized actin declines to near unstimulated values within 20 min (15, 24, 30). Filamentous actin content also increases from 56–72% when Chinese hamster ovary cells adhere to a glass surface, however the time course of this reaction has not been studied (13).

PMN actin polymerization is thought to be regulated by factors which alter the availability of G-actin for filament assembly and by factors which affect the affinity of actin filaments for G-actin. Both actin sequestering and filament capping proteins which can modulate these functions have been purified from these cells (25, 27). Chemoattractants are believed to increase polymerization at the barbed end of actin filaments (3, 15, 30), because polymerization induced by these agents is blocked by cytochalasin D which binds to the barbed end (15, 30). An increase in the number of barbed

end growing sites is observed in cells lysed soon after attractant addition (3).

The transduction mechanisms which control these actin regulatory factors are under investigation in many laboratories. Chemoattractant receptor binding is associated with activation of G proteins which in turn stimulate increased turnover of the phosphoinositol bisphosphate. Actin polymerization stimulated by chemoattractants is inhibited by pertussis toxin treatment of cells. Pertussis toxin catalyzes ADP-ribosylation of 40- and 41-kD GTP binding proteins in PMN, inhibiting receptor stimulated activation of these proteins and the subsequent increase in phosphoinositol metabolism. Recent evidence has shown that micelles of phosphoinositol bisphosphate can stimulate actin polymerization *in vitro* by releasing actin from profilin (18) and gelsolin (16), two proteins which bind to actin and inhibit microfilament assembly. A product of phosphoinositol bisphosphate which increases upon addition of chemoattractant is inositoltriphosphate. This product can increase cytoplasmic Ca^{2+} levels. However previous studies have shown that neither extracellular calcium nor elevation of cytoplasmic calcium levels are required for chemoattractants to increase actin polymerization (23). The transduction mechanisms used by adherence are not yet understood.

Further insight into the mechanisms that mediate PMN actin filament assembly may be achieved by examining changes

1. *Abbreviations used in this paper:* FMLP, formylmethionylleucylphenylalanine; NBD, nitrobenzoxadiazole; PMN, polymorphonuclear leukocytes.

in the arrangement of filamentous actin in PMN. Staining with fluorescently labeled phalloidin or anti-actin antibodies demonstrate a rise in the concentration of F-actin in lamellipodia after addition of chemoattractants (9, 28). Upon attachment to substrate the actin filament network of the cytoplasm adjacent to the substrate increases. In some cases filaments radiate from a number of foci on the lower surface of the cell (2, 5, 8, 13, 22).

We have investigated the effects of adherence to substrate on actin polymerization in PMN using nitrobenzoxadiazole-(NBD) phalloidin and quantitative fluorescence microscopy. Our studies indicate that the time course as well as calcium and functional G protein requirements for adherence-induced actin filament assembly differ from chemoattractant stimulated PMN actin polymerization.

Materials and Methods

PMN Preparation

Human PMN were prepared from 12 ml of whole blood from healthy adult volunteers and were drawn into syringes containing 0.8 ml of EDTA (Sigma Chemical Co., St. Louis, MO). Granulocytes were purified by centrifugation through Fico II-Hypaque solution (Mono-poly resolving media; Flow Laboratories, Inc., McClean, VA) as previously described (24). The granulocyte layer was diluted in 10 vol of 0.15 M NaCl solution and centrifuged at 250 g for 10 min. The resulting cell pellet was then subjected to hypotonic lysis (30 s) to remove residual red blood cells. After centrifugation at 250 g for 10 min, cells were suspended in a modified Hank's buffer (138 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 5 mM NaHCO₃, 0.64 mM Na₂HPO₄ (pH 7.15), 0.66 mM KH₂PO₄, 5.6 mM glucose, 20 mM Hepes). This method yielded 95–98% PMN, the other cells being monocytes and lymphocytes. PMN were purified and stored at room temperature. All experiments were performed within 3 h of venipuncture.

Rabbit PMN were obtained from a 4-h peritoneal exudate (29). The cells were washed twice with saline and then suspended in modified Hank's solution. The cell preparations were ≥ 95% PMN. These cell preparations were used in a number of our nucleation experiments. Rabbit peritoneal PMN responded similarly to human PMN in this assay. No significant differences in nucleation rates were observed in any of our experimental conditions. When exposed to chemoattractant rabbit, peritoneal PMN, like human PMN, responded with an approximately twofold rise in F-actin content (9, 23).

CaCl₂, final concentration of 1 mM, was added to cell suspensions 10 min before cell stimulation in most experiments. For PMN treated with 1 mM EGTA, cells were also preincubated for 10 min before stimulation.

PMN Stimulation and Fixation

1-ml aliquots of cell suspensions (1.5×10^6 cells per sample) were added to 3.5-cm-diam plastic petri tissue culture dishes (Falcon Plastics, Oxnard, CA) and incubated for various times at 37°C. Cells remained viable during the time period of the experiment, > 95% excluding trypan blue after 60 min incubation on the petri dishes. At the completion of the incubation the supernatant containing any nonadherent cells was removed, followed by the addition of 1 ml of buffer warmed to 37°C, which was removed after several gentle swirls to detach any loosely adherent PMN. This buffer was then combined with the first supernatant fluid. The number of cells per milliliter in this solution (number of nonadherent PMN) was determined using a Coulter counter or a hemocytometer. After the addition of 1 ml of buffer to the petri dish, cells were fixed by adding 0.1 ml of 37% formaldehyde and incubating for 15 min at 25°C before staining.

For chemoattractant studies of PMN, L- α -phosphatidyl choline, β -acetyl- δ -O-alkyl (PAF) (Calbiochem-Behring Corp., San Diego, CA), formyl-methionylleucylphenylalanine (FMLP) (Sigma Chemical Co.), and N-formylnorleucylleucylphenyl-alanine (Sigma Chemical Co.) were incubated with PMN before formalin fixation. In some experiments PMNs were incubated for 90 min at 37°C with pertussis toxin (List Biological Laboratories, Inc., Campbell, CA). This highly purified preparation has undetectable adenylate cyclase activity and <0.1% endotoxin contamination. The final concentration of pertussis toxin used in these experiments, 400 ng/ml,

was comparable to that used by previous investigators to inhibit chemoattractant mediated actin filament assembly (23, 24).

Control studies were performed to exclude the possibility that pertussis toxin's inhibitory effects on chemoattractant induced actin filament assembly were secondary to endotoxin or adenylate cyclase activity. When a final concentration of 50 μ g/ml of lipopolysaccharide purified from *Escherichia coli* (10) (a generous gift of Dr. J. Randolph Forehand, Children's Hospital of Philadelphia) was incubated with PMN for 60 min at 37°C, actin assembly was enhanced in response to suboptimal concentrations of FMLP. Stimulation for 60 s with 1×10^{-9} M FMLP resulted in only a 1.14-fold rise in relative F-actin content in cells incubated in buffer (mean of two experiments), while stimulation of PMNs incubated in lipopolysaccharide increased their relative F-actin content by 1.48-fold (mean of two experiments). To examine the effects of adenylate cyclase on FMLP-induced actin assembly, forskolin (final concentration of 30 μ M), an agent known to stimulate adenylate cyclase activity, was mixed with PMN before FMLP stimulation. This reagent had no significant effect on actin filament assembly of suspended PMN exposed to 5×10^{-7} M FMLP for 60 s (2.4-fold rise in F-actin content in untreated PMNs vs. 2.6-fold rise in forskolin treated cells).

NBD-Phalloidin Staining of F-Actin

Staining was performed according to the method of Howard and Oresajo (14). Briefly, 50 μ l of modified Hank's buffer containing 3.46×10^{-6} M NBD-phalloidin (Molecular Probes, Junction City, OR) and 2.1 mg/ml lysophosphatidyl-choline (Sigma Chemical Co.) were added to the fixed cell preparations, mixed, and incubated for 10 min at 37°C. The staining solution was then removed and the adherent cells suspended in 1.0 ml of modified Hank's solution before examination under the microscope. Cells which were fixed immediately after being added to petri dishes (0 min time point) were stained in the petri dishes. Since these cells did not adhere to the petri dish, stain had to be removed by centrifugation at 12,000 g for 1 min in 1.5-ml Eppendorf tubes. The supernatant was removed, cell pellets resuspended in 1.0 ml of modified Hank's solution, and placed again in petri dishes. Just before microscopic examination the buffer was removed, leaving only a thin film of fluid, and a glass coverslip placed on the surface.

Binding of NBD-phalloidin (1.5×10^{-7} M final concentration) to PMN could be competitively inhibited using a 100-fold excess of unlabeled phalloidin (1.5×10^{-5} M final concentration). Addition of unlabeled phalloidin to NBD-phalloidin during staining of unstimulated suspended PMN resulted in a decrease in relative fluorescence from 7.6 to 0.56 as measured by quantitative microscopy (see below). Adherence did not significantly affect nonspecific association of the fluorescent probe. The fluorescence of PMN adhered for 30 min decreased from 13.4 to 0.63 on addition of unlabeled phalloidin.

Quantitative Fluorescence Microscopy

Fluorescence intensities of individual cells were analyzed with a microscope photometer (Zeiss MPM 03). The illumination system consisted of a 100 W Hg lamp, a 40 \times oil immersion objective and an epifluorescence condenser (450–490-nm exciter filter, 510-nm dichroic beam splitter, 520-nm barrier filter). The measuring field was a 20- μ m-diam concentric circle and the illumination field a 30- μ m-diameter. The location of the cell to be measured and focusing were accomplished under dim green illumination from a tungsten lamp. The field was carefully aligned so that only a single cell was exposed. If two cells were partially superimposed or closely adherent, these cells were not measured. We found no significant difference in fluorescence intensity when the plane of focus was varied from the midregion of the cell or slightly above or below the cell (unstimulated cells, midplane focus: 9.51 ± 0.38 fluorescent units; shallow plane of focus: 9.36 ± 0.36 ; deep plane of focus: 9.63 ± 0.32 , 20 determinations). Measured areas were exposed to the fluorescence excitation beam only during the 0.4-s recording period thereby limiting the fading of NBD fluorescence. To further examine the effects of repeated exposure of stained cells to the fluorescence light source, individual PMNs were exposed to light source three times. The mean fluorescence during the first exposure was 10.2 ± 0.2 (SEM, $n = 20$). Each subsequent exposure resulted in a mean decrease in fluorescence intensity of 0.7 ± 0.03 (SEM, $n = 40$). Fluorescence intensity was recorded for 100 cells in each sample. The intensity of unstained cells and background were also measured. Background intensity was subtracted from each value (background and unstained cell intensities were identical, varying from 0.5 to 0.8). Relative F-actin content was calculated by dividing the mean intensity of stimulated PMN by the mean intensity of cells suspended

in buffer. Histograms of cell number vs. fluorescence intensity yielded normal distributions in each experimental condition as assessed by the Kolmogorov-Smirnov one-sample test (19).

Quantitation of F-actin Content by Flow Cytometry

Stained PMN were filtered through nylon mesh and analyzed within 2 h of staining. The intracellular fluorescence was determined by an Ortho Spectrum III (Ortho Diagnostic Systems, Westwood, MA) flow cytometer equipped with an argon laser (488 emission, 500 mw output). All intensities of cellular fluorescence were recorded on the linear scale ranging from 0 to 255 channels. Fluorescent histograms plotting cell number (vertical axis) vs. fluorescence channel (horizontal axis) were recorded for each sample. In all instances histograms yielded a normal distribution (the peak fluorescence channel corresponded to the mean fluorescence). An average of 5,000–10,000 PMN were analyzed per sample. Relative PMN F-actin content was expressed as the ratio of mean fluorescence intensity of stimulated cells to mean fluorescence intensity of PMN in buffer. Unstimulated values were comparable when cells were incubated in modified Hank's buffer or 0.01% DMSO (the highest concentrations used in our experiments) in modified Hank's buffer.

Quantitation Using Methanol Extraction

The method of Howard and Orejo (15) was modified. Adherent PMN were fixed with 3.7% paraformaldehyde for 12–16 h rather than formalin. We found our results were more consistent after paraformaldehyde fixation. Cells were permeabilized and stained as described above. After washing once with buffer, cells were resuspended in 1 ml of absolute methanol (Fisher Scientific Co., Pittsburgh, PA; HPLC grade), macerated vigorously, and incubated for 1 h at room temperature in the dark. Methanol was then removed from the petri dishes, put in 1.5-ml Eppendorf tubes and centrifuged at 12,000 *g* to remove cellular material. The relative fluorescence intensity of the methanol supernatant was then measured using a spectrophotometer (model LS5; Perkin-Elmer Corp., Oakbrook, IL). Excitation and emission wave lengths were 465 and 535 nm, respectively. Slit widths were set at 3 nm. Relative F-actin content was determined by dividing the intensity of methanol extract from adherent cells by the intensity of extract from nonadherent PMN.

Nucleation Assay on Attached Cells

Cells (0.1 ml of 4×10^6 cells/ml) in modified Hank's with or without 0.5% BSA were placed on a 7×16 -mm glass coverslip for various periods of time at 37°C before the coverslip and any unattached cells and fluid were placed diagonally into an acryl cuvette, dimensions $10 \times 10 \times 48$ mm (Sartstedt, FRG) which contained 2.4 ml of $2 \mu\text{M}$ actin in nucleation assay buffer (0.138 mM KCl, 2 mM MgCl₂, 1 mM ATP, 25 mM Tris, pH 8.0, 1 mM EGTA, 0.2% Triton X-100, 0.1% BSA). A 250 μl plug of a 1:1 mixture of parafin and vasaline was solidified in the bottom to position the coverslip in the light path and stabilize its diagonal position. Actin polymerization was followed with the coverslip in the cuvette. In this modification of the method of Conrad and Rink (6) we saw only slight effects due to the presence of the coverslip. By including the medium on the coverslip, we insured that the total cell population was assayed, regardless of how firmly the cells were attached. For the unattached control 0.1 ml of cell suspension was placed directly into a cuvette containing a coverslip.

To insure that nucleation sites attached to the coverslip could be detected, coverslips with attached cells were placed in one cuvette in nucleation assay buffer for several seconds and then removed and placed in a second cuvette. Most of the nucleation activity moved to the second cuvette with the coverslip indicating that the nucleation sites were attached to the coverslip. After the coverslip had been in the cuvette for several minutes, removal of the coverslip resulted in a lowering of the fluorescent signal and a decrease but not an elimination of the subsequent rate of actin polymerization in that cuvette. Thus with time, some of the growing sites were released into the medium. Similar results were obtained whether the coverslip was placed so that the cells were on the side of the coverslip facing the light source or on the opposite side.

To determine if the geometric constraints of our system interfered with detection of fluorescence, the fluorescence intensity of adherent and suspended PMN stained with a fluorescent nuclear stain were compared. PMN adhered to the glass coverslip for 20 min and a comparable number of suspended PMN (1×10^6 /ml) were loaded with a fluorescent nuclear stain (Hooks 33342; Sigma Chemical Co.; loaded by incubation in a $10\text{-}\mu\text{g}/\text{ml}$

solution for 30 min at 37°C) and the relative fluorescence intensities compared (Excitation wavelength 350 nm, Emission wavelength 465 nm). The fluorescence of adherent cells was higher than that of cells in suspension suggesting that a somewhat greater fraction of the adherent PMN were in the path of the light. As observed with pyrene actin, the presence of a glass coverslip had little effect on the fluorescence.

Pyrene Actin

Pyrene actin was prepared from rabbit skeletal actin (20, 26) as previously described (21). The pyrene actin was gel-filtered before use and stored at 4°C as a stock ($20\text{--}30 \mu\text{M}$) in a low salt buffer (5 mM triethanolamine, 1.2 mM ATP, 0.3 mM CaCl₂, 0.1 mM EGTA, and 0.02% Na₃ at pH 7.5). For most experiments $\sim 50\%$ of the actin was pyrene labeled.

Results

Comparison of Microscopic Quantitation of PMN F-actin to FACS

PMN were treated with 5×10^{-7} M FMLP for 30–40 s, formalin fixed, stained with NBD-phalloidin, and then fluorescence intensity measured by fluorescence microscopy or fluorescent activated cell sorter. As shown in Fig. 1, these two methods yielded comparable values for the relative rise in actin filament content induced by FMLP.

Neutrophil F-actin Content Is Increased by Adherence to Substrate

PMN (1.5×10^6 in 1 ml) were incubated at 37°C for various times in plastic petri dishes (tissue culture plastic; Falcon Labware; Oxnard, CA). Cells settled and adhered quickly as determined by analysis of supernatant fluids (see Materials and Methods). Within 30 s $47.1 \pm 4.7\%$ (SEM, $n = 4$ experiments) of PMN had attached to the plastic surface and after 1 min $55.3\% \pm 5.3$ ($n = 5$). By 5 min, $88 \pm 3\%$ ($n = 10$), at 10 min, $95 \pm 2\%$ ($n = 8$), and after 30 min $99 \pm 1\%$ ($n = 11$) of the PMN were adherent (see Fig. 2 A). During this time period PMN gradually spread. At 1 min the mean two-dimensional area of adherent PMN was essentially the same as nonadherent PMN (mean relative area $1.05 \pm .02$ SEM, $n = 50$ cells, fold greater than nonadherent PMN). By 5 min the mean area had increased 4.14 ± 0.09 -fold. By 10 min cells were very flattened, two-dimensional area being almost sixfold greater than nonadherent PMN

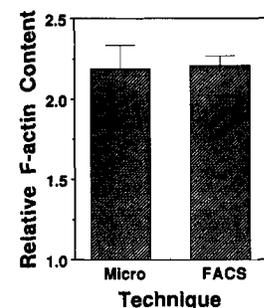


Figure 1. Comparison of fluorescence microscopy to fluorescent activated cell sorter quantitation of F actin content in PMN stimulated with FMLP. PMN 1.5×10^6 cells/ml in suspension were exposed to 5×10^{-7} M FMLP for 30–40 s, formalin fixed, solubilized with lysophosphatidylcholine, and stained with NBD-phalloidin as described in Materials and Methods. One aliquot of fixed and stained cells were allowed to settle on

a plastic petri dish and 100 cells analyzed by microscopy. A second aliquot of the same PMNs was analyzed by fluorescent activated cell sorter, fluorescence of 5,000–10,000 cells being measured. The relative F-actin content was determined for each assay as described in Materials and Methods. Bars represent the SEM of eight experiments.

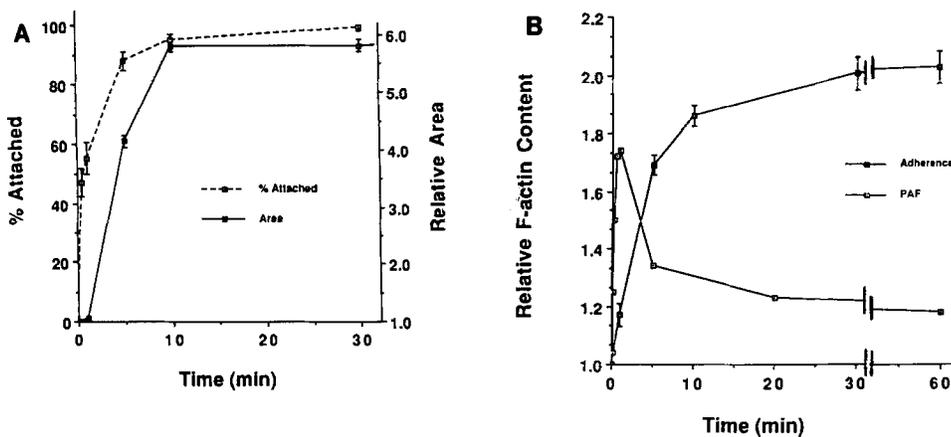


Figure 2. (A) Time course of PMN attachment to and spreading on a plastic surface. Cells were allowed to settle at various times and after gentle washing the percent of total cells attached, determined as described in Materials and Methods (---, □). The earliest time point measured was 30 s. At the same times beginning at 1 min, additional plastic dishes containing PMN were gently washed and overlaid with formalin fixative (3.7%). The mean of the two-dimensional areas of 50 adherent PMNs was determined for each time point using phase micro-

scopy and an Image-I/AT image analyzer. These mean areas were divided by the mean of the two-dimensional areas of nonadherent cells to give relative area values (—, ■). The bars represent the SEM. Over 10 min the mean two-dimensional cell area increased nearly sixfold (5.74 ± 0.10 SEM). (B) Time course of actin filament assembly after PMN attachment to a plastic surface compared to suspended PMN exposed to an optimal concentration (1×10^{-8} M) of platelet activating factor (PAF). PMN were allowed to adhere to plastic culture plates for various times, fixed, permeabilized, and stained with NBD-phalloidin as described in Materials and Methods. Fluorescence intensity of 100 cells were determined and a mean fluorescence intensity determined for each time point. The bars represent the SEM. The number of experiments at each time point are reported in Results. For comparison F-actin content of suspended PMN after exposure to PAF at various times is plotted. Cells were treated fixed and stained as described for adherent cells. Each point represents the mean fluorescence intensity of 5,000–10,000 PMN measured by fluorescent activated cell sorter. Peak F-actin content was observed within 30–60 s.

(5.74 ± 0.10). Similar relative increases in area were also observed at 30 (5.74 ± 0.12) and 60 (6.11 ± 0.15) min.

In association with adherence and spreading, a rise in F-actin content was observed (see Fig. 2 B). After 1 min the relative F-actin content of adherent PMN had increased to 1.17 ± 0.04 ($n = 4$ experiments) times that of cells in suspension. Within 5 min the F-actin content was 1.69 ± 0.03 (SEM, $n = 8$) fold higher than suspended cells. The concentration of F-actin continued increasing to 1.86 ± 0.04 (SEM, $n = 12$) at 10 min, reaching a maximum by 30 min, 2.00 ± 0.06 (SEM, $n = 21$), persisting for 60 min, 2.02 ± 0.06 (SEM, $n = 4$) (the longest time point studied). The rise in F-actin content closely paralleled the rate of spreading (compare solid squares in Fig. 2, A and B).

The increase in F-actin induced by adherence was slower than that seen after addition of chemoattractants and persisted for a more prolonged period (Fig. 2 B, compare ■ with □). While chemoattractant stimulated PMN achieved a maximum F-actin content by 30–60 s stimulation, adherent PMN had not reached maximal levels of F-actin content by 5 min. Continued stimulation with chemoattractant was associated with a decrease in F-actin content to nearly baseline levels (between 5 and 20 min) while PMN exposed to the adherent surface for more prolonged periods maintained an elevation in F-actin content, a twofold rise persisting for 60 min.

The rise in F-actin associated with adherence was temperature dependent, a less dramatic rise in F-actin being observed at 25°C as compared to 38°C (after 30 min 1.6 at 25°C vs. 2.0 at 38°C). The extent of actin filament assembly also varied with the substrate to which cells were attached. The magnitude of the rise in F-actin was less prominent when human neutrophils were adhered to glass (maximum rise 1.3). When PMN were attached to plastic culture dishes in buffer containing 1 mg/ml of lipid-free BSA, the rise in F-actin was lower than cells attached in buffer without albumin (after 30

min, 1.5 in albumin vs. 2.0 in buffer without albumin). A greater increase in F-actin was observed in human neutrophils than in rabbit peritoneal exudate neutrophils (maximum rise 1.6). Incubation of human cells in 5% CO_2 atmosphere resulted in similar values. The majority of experiments were performed under conditions which resulted in maximum rises in F-actin content i.e., human PMN adhered to tissue culture plastic petri dishes in modified Hank's buffer.

Addition of chemoattractant to cells attached to substrate for 5 and 10 min resulted in a further increase in the amount of actin polymerized. The effect of chemoattractant was most dramatic after short settling times, 5 min, and was reduced in cells that had been allowed to attach for 10 min. Cells allowed to adhere to tissue culture plastic for 30 min demonstrated no further rise in F-actin content after stimulation with the chemoattractant (see Fig. 3).

The rise in F-actin content was also quantitated by methanol extraction of NBD-phalloidin stained adherent PMN. As measured by this method, PMN allowed to adhere demonstrated a comparable rise in F-actin after 30 min, $1.99 \pm .29$ (SEM, $n = 4$). Although a rise in F-actin was observed in all experiments, values were less reproducible than those determined by the microscopic assay, therefore most experiments were analyzed using the fluorescent microscope.

Adherence Induced Changes in Actin Nucleation Sites

To determine if the changes in actin polymerization correlated with changes in the nucleation activity of extracts, nucleation from lysates of attached cells was monitored using a modification of the method of Conrad and Rink (6). Cells attached to a coverslip for various lengths of time were lysed as the coverslip was placed diagonally into the cuvette containing 0.2% Triton and $2 \mu\text{M}$ pyrene actin added just before placement of the coverslip (see Materials and Methods). In this assay, the initial rate of polymerization of the pyrene ac-

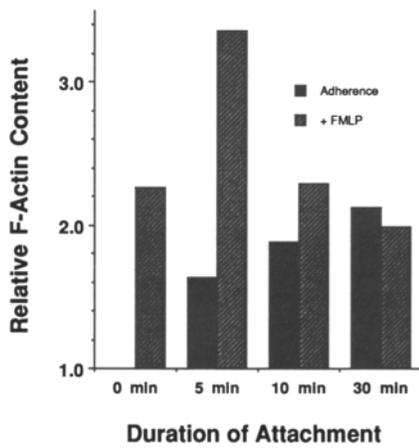


Figure 3. Comparison of F-actin content of adherent and nonadherent cells after exposure to the chemotactic agent FMLP. Duplicate aliquots of PMN were allowed to adhere for 5, 10, or 30 min. On completion of the adherence incubation time, the supernatant of one sample of each set was quickly removed and replaced with buffer containing FMLP (final concentration 5×10^{-8} M). All cell samples were fixed and stained 40 s later as described in Fig. 1. The far left bar represents the relative F-actin content of suspended PMN exposed to FMLP for 40 s. For each experimental condition the mean fluorescence intensity of 100 cells was determined. Each value represents the mean of two experiments.

tin is proportional to the number of actin nucleation sites present in the lysate (3).

Fig. 4 shows nucleation activity of rabbit PMN in suspension and attached to coverslips for various times. In >65 experiments as compared to suspended PMN, we detected no significant increase in the nucleation activity of cells attached to coverslips for 10 s up to 30 min. By 5 min a consistent decrease in nucleation rate was observed in adherent

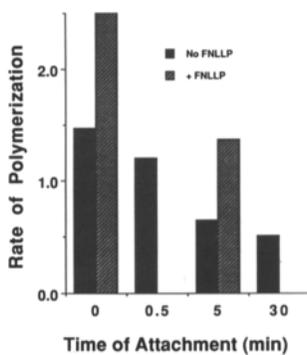


Figure 4. Nucleation activity of PMN after adhering to glass coverslips. A representative experiment comparing nucleation rates of suspended and attached rabbit PMN. The nucleation rates of PMN (0.1 ml of 4×10^6 /ml) were measured as described in Materials and Methods. PMN were mixed immediately into the fluorimeter cuvettes containing the Triton solution and $2 \mu\text{M}$ pyrene actin (0 min adherence, ■) or allowed to attach to glass coverslips for 0.5, 5, or 30 min (■) before placement in the cuvettes. ▨ represent PMN in suspension or attached to glass for 5 min which were then treated with 1×10^{-7} M FNLLP for 10 s before lysis. The rates of actin assembly are indicated on the vertical axis in relative fluorescence units per minute. Values represent the mean of two duplicate samples. Values for the two determinations in each experimental condition were nearly identical (0 min: 1.5 and 1.45 U/min; 0 min + FNLLP: 2.50 and 2.60 U/min; 30 s: 1.15 and 1.25 U/min; 5 min: 0.65 and 0.65 U/min; 5 min + FNLLP: 1.40 and 1.35 U/min; 30 min: 0.50 and 0.51 U/min).

PMN as compared to cells in suspension. Continued attachment was associated with a further decrease in the number of nucleation sites in the attached cells. Adherent cells could be induced to increase nucleation activity by addition of 10^{-7} M *N*-formylnorleucylphenyl-alanine indicating that changes in nucleation activity could be detected on coverslips (see hatched bar, 5 min adherence time point in Fig. 4).

Effects of Pertussis Toxin

Incubation of neutrophils in 400 ng/ml of pertussis toxin for 90 min had no effect on the adherence-associated actin polymerization and did not reduce the number of adherent PMN (99–100% adherent after 30 min). The same population of pertussis toxin-treated cells did not increase their actin levels upon stimulation in suspension with the chemoattractant FMLP (see Fig. 5).

Effects of Calcium

Removing calcium from the extracellular medium did not prevent cell attachment. The percentage of cells attached to the plastic culture dishes (5 min $93.5 \pm 2.5\%$ attached, SEM, $n = 5$; 10 min $95.3 \pm 2.8\%$, $n = 4$; 30 min $99 \pm 0.4\%$, $n = 5$) did not differ significantly from neutrophils allowed to attach in calcium-containing medium (see above). With the exception of a small percentage of PMN which remained rounded in some EGTA experiments, cell spreading was also comparable in the two buffer conditions (see Fig. 9). However, removal of calcium did markedly reduce the rise in F-actin content (see Fig. 6). The effects of calcium and EGTA on adherent PMN actin filament content were fully reversible if the medium was changed within 5 min of attachment. When Ca^{2+} containing buffer was replaced by EGTA buffer after 5 min of adherence and PMN incubated for an additional 25 min, the F-actin content, 1.16 ± 0.04 (SEM, $n = 3$ experiments), was comparable to cells adhered in EGTA alone, 1.08 ± 0.08 ($n = 3$). Similarly PMN sequentially adhered in EGTA buffer followed by Ca^{2+} buffer had an F-actin content, 1.72 ± 0.1 ($n = 3$), which was comparable to PMN incubation in Ca^{2+} buffer alone, 1.64 ± 0.05 ($n = 3$). If PMNs were allowed to adhere in either ionic condition for a more prolonged period, 15 min, no shift in F-actin content could be induced by changing the buffer conditions.

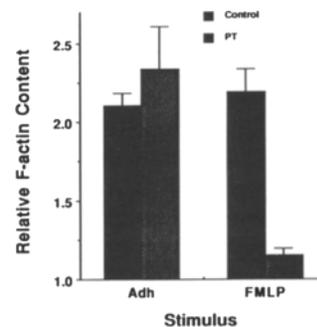


Figure 5. The effects of pertussis toxin on adherence and chemotactic agent mediated actin filament assembly. The black bars represent relative F-actin content of PMN incubated in buffer for 90 min and then allowed to adhere for 30 min (left) or exposed to a final concentration of 5×10^{-8} FMLP for 40 s (right) before fixation and staining. ▨ represent relative F-actin content of

a second sample of the same PMN incubated for 90 min in a final concentration of 400 ng/ml pertussis toxin before adherence or exposure to FMLP. The brackets represent the SEM of three experiments.

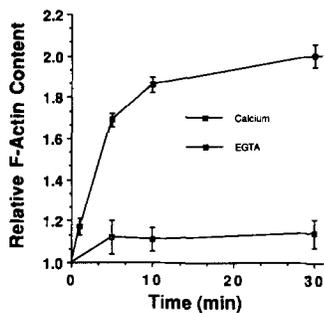


Figure 6. Comparison of F-actin content of PMN allowed to adhere in a Ca^{2+} containing and Ca^{2+} -depleted buffer. The lower curve represents the F-actin content of PMN allowed to adhere to a plastic surface for various times in modified Hanks/Hepes buffer containing 1 mM EGTA. PMN were preincubated for 10 min at 37°C in the EGTA containing

buffer before allowing them to settle on petri dishes. The brackets represent the SEM of $n = 2$ (5 min), 5 (10 min), and 8 (30 min) experiments. The upper curve demonstrating F-actin content in Ca^{2+} -containing buffer is copied from Fig. 2 for comparison. All of the EGTA experiments were performed in parallel with studies in calcium containing buffer.

Effects of Cytochalasin

Cytochalasin completely blocked the rise in F-actin content induced by adherence. Cells allowed to attach to substrate in the presence of cytochalasin had slightly lower levels of F-actin than control cells in suspension (Fig. 7). Cytochalasin had little effect on F-actin content in unstimulated cells in suspension (relative fluorescence of control neutrophils: 11.5 ± 0.7 vs. 11.7 ± 0.6 for cells treated with $2 \mu\text{g}$ cytochalasin B). Concentrations of cytochalasin B which reduced actin filament assembly did not impair attachment to the substrate, but did inhibit spreading. Addition of cytochalasin to neutrophils attached to substrate for 30 min resulted in the depolymerization of adhesion-induced F-actin. Within 10 min in $2 \mu\text{g}/\text{ml}$ cytochalasin B, F-actin levels declined to that of unattached cells. Similar effects were observed when PMN were treated with cytochalasin D. Exposure of PMN to 0.05, 0.1, and $0.2 \mu\text{g}/\text{ml}$ of cytochalasin D 1 min before and for 30 min during adherence was associated with a dose dependent de-

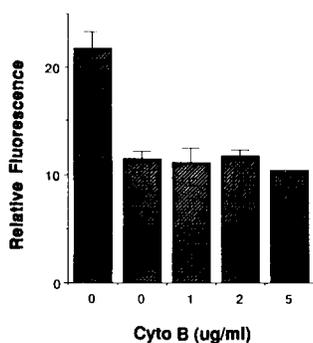


Figure 7. The effects of cytochalasin B on adherence induced PMN F-actin content. PMN were exposed to various concentrations of cytochalasin B for 10 min before being allowed to adhere to plastic petri dishes for 30 min, fixed, and stained as described in Fig. 2. The far left bar represents the F-actin content (relative fluorescence) of untreated PMN allowed to adhere for 30 min.

The next bar represents the F-actin content of untreated and unstimulated PMN. Cytochalasin B ($1\text{--}5 \mu\text{g}/\text{ml}$) blocked adherence induced actin filament assembly, but did not decrease F-actin content below that of unstimulated PMN. Brackets represent SEM of $n = 5$ (adherent untreated PMN), $n = 5$ (nonadherent, untreated PMN), $n = 3$ (adherent, $1 \mu\text{g}/\text{ml}$ cytochalasin B), $n = 4$ (adherent, $2 \mu\text{g}/\text{ml}$ cytochalasin B). Adherence in the presence of $5 \mu\text{g}/\text{ml}$ cytochalasin B was performed once. Since the fluorescent light source varies in intensity from day to day, for comparison between experiments all values were corrected to a known standard.

crease in F-actin content (respectively, 27%, 60%, and 71% reduction in F-actin content).

Localization of Actin in Adherent Cells

Cells fixed after 5 min of adherence and stained with NBD-phalloidin or rhodamine-phalloidin showed F-actin concentrated in lamellipodia. After 30 min of adherence, ruffling regions were still present and in addition, a number of small foci of F-actin were now visible on the lower surface of the cell (Fig. 8). In the absence of extracellular calcium the small foci of fluorescence were less apparent; F-actin was concentrated in some of the peripheral lamellipodia. Despite the marked differences in F-actin content, the morphology of adherent PMNs viewed by phase microscopy were similar in the presence or absence of calcium (Fig. 9).

Discussion

Fluorescence microscopy of NBD-phalloidin-stained cells has been used to quantitate actin filament assembly in human PMN as they attach to a surface. These studies demonstrate that adherence is associated with a slow rise in F-actin, maximal levels being observed after ~ 10 min. The majority of PMN were attached to the surface for a prolonged period (>5 min) before the maximum rise in F-actin content was observed. This slow rate of actin assembly may be primarily caused by a gradual activation or clustering of adherence receptors. A more likely explanation however is suggested by the close correlation between the rate of spreading and the rate of rise in F-actin content (Fig. 2, A and B). As PMN spread, additional adherence receptors would be expected to come in contact with the plastic surface and stimulate a continued rise in F-actin content.

In addition to a slow rate of actin assembly, unlike continued chemoattractant stimulation which is associated with actin disassembly and a decrease in F-actin content, PMN exposed to an adherent surface for a prolonged period maintain an elevated F-actin content. A twofold rise in F-actin level persists for at least 60 min. This finding suggests a possible stabilization of actin filaments in association with prolonged adherence. This stabilization may reflect differences in the activity of one or more actin binding proteins in adherent as compared to chemoattractant stimulated PMN.

While an increase in the number of barbed-end nucleation sites is seen at the time of actin polymerization induced by chemoattractants (3), little or no increase in nucleation sites is observed during the polymerization induced by adherence. It is possible that a small rise in the number of nucleation sites occurs but is too small to be detected. The insignificant change in nucleation rate is consistent with the relatively slow rate of polymerization induced by adherence as compared to chemoattractant. In addition albumin-coated glass coverslips were used in our nucleation assay rather than plastic coverslips which interfered with fluorescence measurements. The use of a glass substrate may have further impaired our ability to detect a rise in nucleation sites since maximal PMN actin assembly would not be expected under this condition. PMN spreading and strength of attachment have been shown to vary with the substrata and protein or proteins present (16A). Similarly we find that the adherence induced rise in PMN F-actin is highly dependent on these factors.

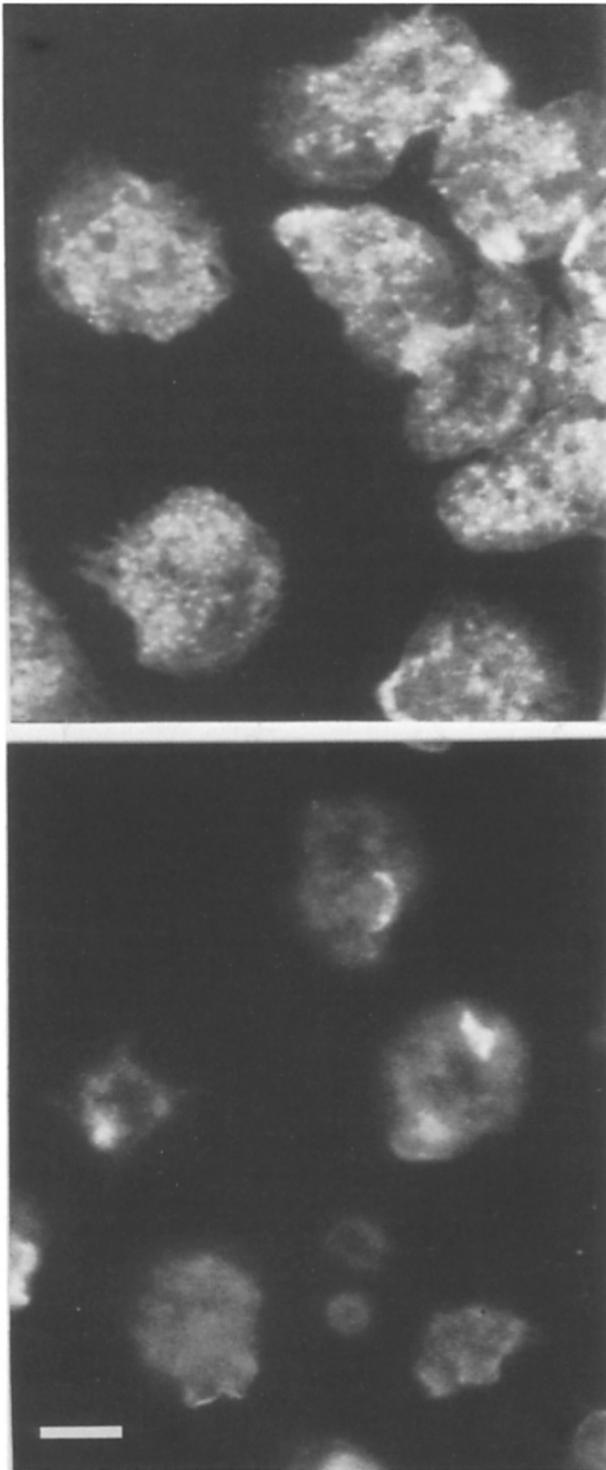


Figure 8. Fluorescent micrographs of NBD-phalloidin-stained adherent PMN in the presence and absence of calcium. PMN were allowed to adhere to plastic petri dishes for 30 min, fixed permeabilized, and stained with NBD-phalloidin as described in Materials and Methods. (*Top section*) PMN allowed to adhere in calcium-containing buffer. (*Bottom section*) PMN adhered in calcium-depleted buffer. Cells were incubated in EGTA buffer as described in Fig. 6. The majority of PMN in the low calcium condition demonstrated spreading which was comparable to PMN in calcium, however a small percentage did remain rounded. The mean fluorescence of duplicate samples was quantitated as described in Fig. 2. In this experiment, PMNs adhered in calcium demonstrated a 2.04-fold rise in F-actin content as compared to nonadherent PMN, while PMN adhered in EGTA buffer had a mean F actin content 1.0. Bar, 10 μ m.

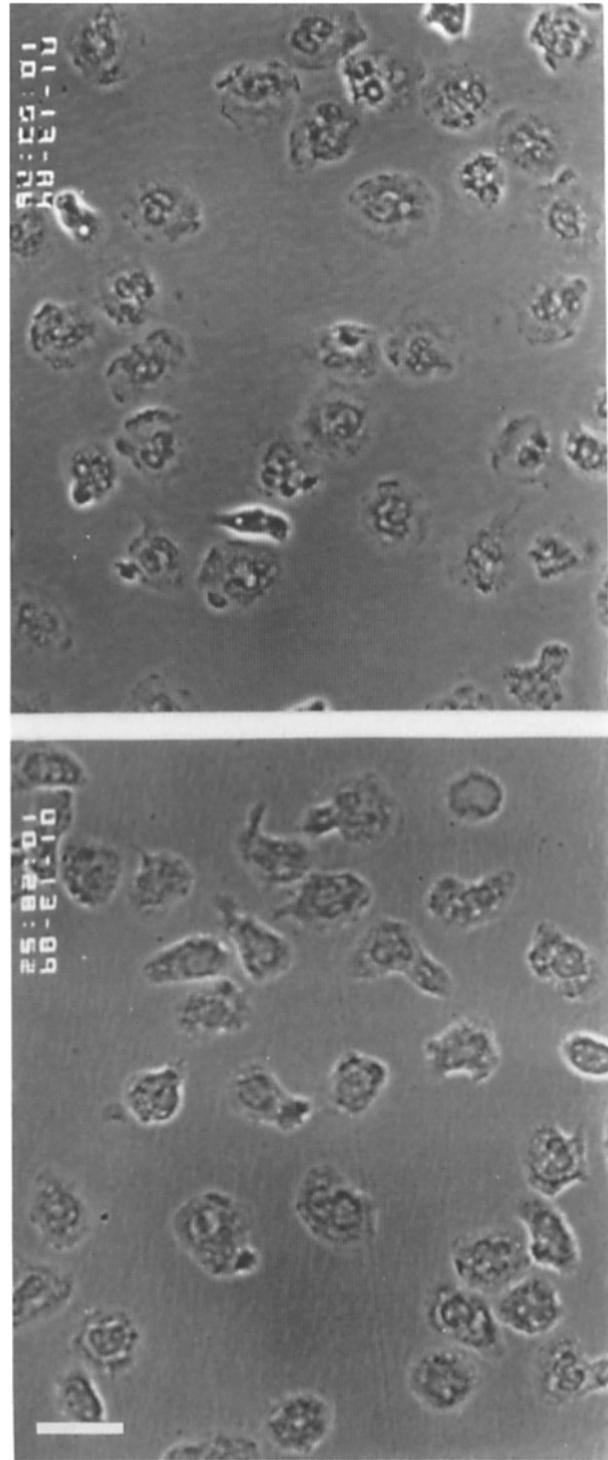


Figure 9. Phase micrographs of adherent PMN in the presence and absence of calcium. Experimental conditions as well as fixation and staining methods were identical to Fig. 8. (*Top section*) Calcium-containing buffer. (*Bottom section*) Calcium-depleted buffer. The relative F-actin contents of PMN adhered in the presence and absence of calcium were comparable to Fig. 8. Bar, 30 μ m.

Our fluorometric assay system is capable of detecting increases in nucleation activity induced by FMLP stimulation of attached cells. In addition comparable intensity values for fluorescent nuclear staining of suspension and adherent PMN are observed indicating that the low levels of nucleation activity observed during adherence are not secondary to

any geometric constraints of our assay system. In fact, the attached cells seem to be somewhat more efficiently sampled in the fluorimeter than suspension cells. Thus the decrease in nucleation upon attachment may be even greater than suggested by our data.

The actin polymerization induced by adherence does appear to occur at the barbed end of filaments because it is blocked by cytochalasin (7). Cytochalasin also results in a rapid depolymerization of adherence-induced actin filaments returning F-actin content to unstimulated values. Similar cytochalasin effects have been seen in PMN stimulated with chemoattractants (30) and in thrombin stimulated platelets (4, 11). These findings suggest that the increase in F-actin caused by both adherence and chemoattractants depends on the presence of free barbed actin filament ends.

Pertussis toxin-inhibited G proteins are not required to initiate adherence-induced actin filament assembly, however adherence-associated assembly does require extracellular calcium. In calcium-free media actin filament assembly is markedly reduced while there are no clear differences in number of cells which attach and spread. It has recently been observed in human PMN that attachment is associated with a rapid rise in intracellular ionized calcium (17). A depression in this rise has been associated with defective cell spreading. Cell spreading is observed in our experiments suggesting that some rise in intracellular Ca^{2+} may occur. Extracellular calcium may play a role in adherence of specific molecules to the substrate or a transmembrane calcium flux may be needed for adherence-induced actin filament assembly.

Chemotactic peptide stimulation of PMN in the early stages of adherence (≤ 5 min) results in an additive rise in F-actin content, the rise in concentration being greater than threefold. In contrast simultaneous stimulation with optimal concentrations of two chemoattractants (PAF and FMLP) fails to induce any additive rise in F-actin content (24). The results of peptide stimulation of cells spreading on substrate supports our conclusion that attachment and chemoattractants use different transduction mechanisms to induce actin filament assembly. More prolonged adherence decreases the rise in F-actin induced by the chemoattractant. At these times we also see a decrease in the nucleation activity of attached cells.

Photomicrographs of fluorescent phalloidin-stained adherent PMN reveal many small foci of F-actin along the lower surface of the cells. Similar F-actin foci have been observed in adherent macrophages (1). Electron micrographs of the adherent surfaces of PMN (2) and macrophages (1, 12) reveal similar globular foci containing radiating microfilaments which assemble in response to adherence, concurrent with cell spreading. The F-actin foci seen in adherent cells may represent actual attachment sites (28). In preliminary studies these sites do not stain with anti-vinculin or antitactin (generously provided by Dr. Joann Otto, Purdue University).

The actin polymerization stimulated by adherence differs from that induced by chemoattractants in a number of aspects: (a) The rise in F-actin content is sustained. (b) Pertussis toxin does not block the F-actin increase. (c) Removal of calcium from the medium does block the increase. (d) At early times of adherence, the addition of chemoattractant results in an additive increase in F-actin. (e) The F-actin is

localized in small foci on the lower surface of the cell as well as in the lamellipodia and cortex. These observations indicate that chemoattractants and adherence act by different transduction steps to activate PMN actin filament assembly.

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