Modulation of the cytoskeleton and intracellular calcium in leukocytes exhibiting a cancer-associated chemotaxis defect

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Abstract: Monocyte chemotaxis is severely depressed in patients with advanced tumors, but the cellular basis for this chemotactic defect is not known. Because the actomyosin cytoskeleton is thought to play a primary role in chemotaxis, we have employed flow cytometry to examine several aspects of the contractile machinery including myosin II, myosin light chain kinase (MLCK), actin, and cytoplasmic calcium in unstimulated and in formylpeptide-stimulated neutrophils and monocytes. Serum-pretreated polymorphonuclear leukocytes (PMNs) and monocytes from healthy blood donors or PMNs and monocytes isolated from tumor patients were studied. Leukocytes pretreated with serum from cancer patients exhibited decreased baseline myosin staining and a vastly different response to formylpeptide stimulation compared with leukocytes pretreated with normal human serum. In contrast, similar amounts of MLCK were observed in neutrophils and monocytes preincubated with normal or cancer serum with or without stimulation with formylpeptide. The fluorescent calcium indicator fluo-3 showed that resting and fMLP-stimulated levels of intracellular calcium were not significantly different in control and cancer serum-pretreated human leukocytes or in leukocytes isolated from tumor patients. Similarly, resting and fMLP-stimulated levels of F-actin in cancer patients' leukocytes as assessed by NBD-phallacidin staining did not differ significantly from those of normal leukocytes. Because the actomyosin cytoskeleton is intrinsically involved in leukocyte chemotaxis, alterations in the cytoskeleton may dramatically affect cell motility. The cytoskeletal alterations and changes in the response of leukocytes pretreated with cancer patients' serum to formylpeptide stimulation as described here may result in decreased chemotaxis by these cells. J. Leukoc. Biol. 54: 351-359; 1993.

Key Words: actin • myosin • calcium • flow cytometry • cancer • chemotaxis

INTRODUCTION

Chemoattractant stimulation of neutrophils and monocytes induces rapid alterations in cell morphology that are a prerequisite for cell locomotion. Dynamic remodeling of the actomyosin cytoskeleton is thought to play a prominent role in these structural changes. In leukocytes and single-cell eukaryotes such as Dictyostelium discoideum and Acanthamoeba castellani, cytoplasmic actin and myosin* have been studied extensively and are known to generate the forces that drive chemotaxis [1, 2]. Within seconds of exposure to a chemoattractant, extensive actin polymerization occurs, followed over the next few minutes by much slower depolymerization [3, 4]. Concurrently, actin-rich pseudopods are extended by the cell and, in time, the entire cell becomes polarized structurally with actin concentrated in both the lamellipod and uropod [4-7]. The polymerization and depolymerization of a three-dimensional actin lattice may be responsible for the cytoplasmic protrusive and contractile activities associated with cell locomotion [2]. Actin polymerization may occur in the absence of intracellular calcium [8], but breakdown of the actin meshwork is promoted by elevated calcium levels through activation of certain actin-binding proteins [2, 9]. Thus, formation and remodeling of the actin cytoskeleton are regulated by cytoplasmic calcium and by a range of known actin-binding proteins including myosin II, filamin, acumentin, profilin, gelsolin, and α-actinin [2, 10, 11].

Subsequent to chemotactic activation and concurrent with actin remodelling, myosin heavy chain is transiently dephosphorylated and myosin light chain is phosphorylated [12, 13], triggering myosin heavy chain assembly into thick filaments [2, 14, 15]. Following this, myosin heavy chain phosphorylation occurs, impeding further assembly of myosin thick filaments [16, 17] and leading to depolymerization of existing myosin filaments [18].

Myosin I, a type of myosin that does not form filaments, has been localized at the leading edge of motile D. discoideum and in the contractile ring of dividing amebas [19, 20], but has not yet been found in vertebrate leukocytes [21]. Thus, the importance of myosin I in the motility of these cells is unknown, whereas myosin II has been much more thoroughly characterized in mammalian leukocytes. While colocalized in the uropod and lamellipod of vertebrate non-muscle cells, it is thought that myosin thick filaments may interact with actin by means of myosin ATPase to generate the forces required to drive cell migration and assist in tail retraction [15, 22]. These actomyosin interactions are regulated by calcium [23] and by the phosphorylation of the myosin light chain by myosin light chain kinase (MLCK) [24-26]. Upon stimulation with chemoattractant, intracellular calcium concentrations rise rapidly (10- to 50-fold) into the micromolar range [27, 28]. Initially, additional free calcium is derived from an intracellular storage site, and this is

Abbreviations: ATPase, adenosine triphosphatase; BSA, bovine serum albumin; CA, cancer; CT, control or normal; DMSCO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; fMLP, N-formylmethionyl-leucyl-phenylalanine; HEPES, N,2-hydroxyethylpiperazine-N,2'-ethanesulfonic acid; HBS, HEPES buffered salt solution; MCN, mean channel number; MLCK, myosin light chain kinase; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte.

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*Unless otherwise stated, "myosin" refers to myosin II or conventional myosin.
further augmented by a later influx of extracellular calcium [29]. This increase in intracellular calcium is transient; efflux of calcium begins within seconds, and intracellular calcium returns to prestimulation levels within 10 min.

It is presumed that the activation of myosin and actin in conjunction with these calcium fluxes is necessary to bring about normal chemotaxis and that alterations in any of the steps of this sequence may result in defective or reduced chemotaxis. A severe defect in monocyte chemotaxis has been demonstrated in many cancer (CA) patients, but the cause of this defect is not known [30, 31]. Monocytes, macrophages, and neutrophils are thought to participate directly in tumor destruction [32, 33], and studies of tumor models in experimental animals have consistently demonstrated that these leukocytes are potent antitumor effectors [34]. Monocyte and neutrophil accumulation at sites of tumor growth is accomplished by means of chemotaxis and this response appears to be an essential aspect of the antitumor response [35]. Because of the significance of the leukocyte cytoskeleton in chemotactic activation, we have examined the myosin II and MLCK content, actin polymerization, and intracellular calcium fluxes in neutrophils and monocytes from normal (CT) individuals and CA patients and in CT and CA serum-pretreated normal neutrophils and monocytes. Unstimulated and chemotactic-stimulated cells were studied in both of these groups. No differences were evident between the CT and CA groups with respect to intracellular free calcium fluxes or actin polymerization in response to chemotactic stimulation. However, compared with PMNs and monocytes pretreated with CT serum, unstimulated and fMLP-stimulated leukocytes pretreated with serum from CA patients exhibited significantly altered levels of myosin staining. This abnormal modulation of myosin in CA patients' leukocytes may contribute to the chemotactic defect observed in these patients.

METHODS AND MATERIALS

Human leukocytes

Peripheral venous blood samples were obtained from healthy adult volunteers and from patients admitted to Cook County Hospital for diagnosis and treatment of primary head and neck tumors. Patients included in this study were not receiving chemotherapy, radiotherapy, or medication at the time of sample collection. Blood was collected by venipuncture in sterile EDTA-containing tubes and leukocytes were isolated by a modification of the method of Boyum [36]. Erythrocytes were gravity-sedimented at room temperature by the addition of pyrogen-free dextran (200 kD) to a final concentration of 1.25%. The leukocyte-rich plasma was diluted with an equal volume of HEPES-buffered saline (HBS), pH 7.4, containing 140 mM NaCl, 10 mM KCl, 10 mM N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES), 5 mM glucose, and 2 mg/ml bovine serum albumin (BSA) and supplemented with 2 mM ethylenediaminetetraacetic acid (EDTA). This suspension was layered onto a cushion of Lymphocyte Separation Medium (Organon Teknika, Durham, NC) and centrifuged at 500g for 5 min at room temperature. Mononuclear cells at the interface of the discontinuous gradient were collected, diluted with HBS containing EDTA and 2.5% dextran, and centrifuged at 250g for 5 min at room temperature. The platelet-rich supernatant was removed and this washing procedure repeated twice. The neutrophil pellet was resuspended in HBS with EDTA and centrifuged at 6000g in a microcentrifuge for 2 s at room temperature. Contaminating erythrocytes were removed by brief hypotonic lysis and the leukocytes washed three times in HBS with EDTA. Monocytes and neutrophils were finally resuspended in HBS containing 0.2 mM CaCl$_2$ and 1 mM MgSO$_4$ and kept on ice until use.

Serum pretreatment

Blood samples from healthy adult donors and patients with head and neck tumors were collected by venipuncture and allowed to clot overnight at 4°C. The serum was collected by centrifugation, and aliquots were stored frozen at -20°C until use. Purified human peripheral blood neutrophils or monocytes were pretreated by incubation with 10% human serum in HBS with EDTA at 37°C for 30 min in siliconized glass test tubes. The serum was removed by centrifugation and the cells were resuspended in HBS at 4°C. It should be noted that human serum contains C5a, an intermediate breakdown product of the complement cascade, which is also a potent chemoattractant for human neutrophils and monocytes [37]. However, C5a is extremely labile in whole serum and is degraded rapidly on storage at -20°C, so it is unlikely that serum-pretreated leukocytes were exposed to C5a during the pretreatment.

Chemotaxis assays

N-Formylmethionyl-leucyl-phenylalanine (fMLP; Peninsula Laboratories, Belmont, CA) in concentrations ranging from 0.1 to 100 nM was placed in the bottom wells of a 48-well chemotaxis chamber (Neuroprobe, Bethesda, MD) and covered with a 5-μm pore size polivinylpyrrolidone-free filter (Nuclepore, Pleasanton, CA). Control and CA serum-pretreated human neutrophils or monocytes (2·10$^4$ cells) were loaded into the upper wells and the chambers were incubated at 37°C for 2 h. The filters were removed, fixed in methanol, stained, rinsed, dried, and mounted on glass slides in Permount (Fisher Scientific, Itasca, IL). Assays were quantitated by counting the number of cells in five contiguous 40x fields of a Nikon Optiphot microscope (Nikon, Garden City, NY).

Flow cytometry

Samples analyzed on the flow cytometer were first filtered through a 20- to 40-μm mesh nylon filter (Fisher Scientific) to remove cell clumps and kept in the dark on ice until use. Cells were analyzed on an Epics V flow cytometer (Coulter Electronics, Hialeah, FL) interfaced to a Coulter MDADS data acquisition system. Fluorescence was excited at 488 nm with 400-mW laser output and detected using a combination of a laser blocking filter with 560-nm short-pass dichroic and 525-nm bandpass filters. Polystyrene spheres (Immunochrome; Fine Particle Division of Coulter Electronics) were used for fine adjustment of the laser beam. Monocytes and neutrophils were gated on the basis of light-scattering characteristics (forward angle vs. 90° light scatter). Histograms of fluorescence intensity on either a logarithmic or linear scale versus cell number or time were collected for a minimum of 5000 cells per sample.

Some cells were fixed in 2% buffered paraformaldehyde and stained with either phycoerythrin-conjugated MY-9 (Coulter Immunology, Hialeah, FL; diluted 1:30) or phycoerythrin-conjugated nonspecific mouse immunoglobulin (diluted 1:30). MY-9 binds to normal peripheral blood monocytes but not to granulocytes, erythrocytes, platelets, or lymphocytes [38] and was used to aid in determining the ex-
act monocyte and neutrophil gate settings and to ascertain the proportion of monocytes counted in the monocyte gate.

Myosin and MLCK staining procedures

Cells were exposed to either buffer alone or fMLP for 4 min at 37°C. For PMNs, a uniform field of 1 nM fMLP was employed as stimulant, whereas 0.1 nM fMLP was used for monocytes. Anti-myosin and anti-MLCK antibodies were the generous gift of Dr. P. deLanerolle (University of Illinois at Chicago) and were used as described previously but with some modifications [39]. Unstimulated or fMLP-treated cells were fixed at 4°C in 2% paraformaldehyde in phosphate-buffered saline (PBS), washed, and then lysed in acetone (−20°C). After washing in 50% ethanol, autofluorescence and unreacted fixative were quenched using sodium borohydride (1 mg/ml) in 50% ethanol. Cells were then rinsed in PBS containing 1% normal goat serum and 0.1% BSA and nonspecific binding was blocked using 5% normal goat serum. Cells were then reacted with primary antibody for 1 h at room temperature. Either rabbit anti-human platelet myosin serum (1:40 dilution) or affinity-purified rabbit anti-turkey gizzard MLCK (1:100 dilution) was used as the primary antibody. Anti-human platelet myosin antiserum has broad reactivity with both myosin heavy and light chains, reacts strongly with mammalian and avian nonmuscle myosin, and reacts to a lesser degree with mammalian and avian smooth muscle myosin [40]. Polyclonal rabbit anti-turkey gizzard MLCK antibody reacts with MLCK of smooth muscle but not with actin, myosin, tropomyosin, α-actinin, or filamin [39]. After this incubation, cells were washed and further incubated with fluorescein isothiocyanate–goat anti-rabbit immunoglobulin G (1:60 dilution; Cappel) for 1 h. Cells were then washed using three changes of PBS. All buffers and antibodies were passed through a 0.22-μm pore size micropore filter before use to remove aggregates.

F-actin content

Human peripheral blood PMNs and monocytes from healthy CT subjects, PMNs and monocytes from CA patients, or serum-pretreated CT leukocytes were stimulated in suspension with either 1 to 100 nM fMLP (for PMNs) or 0.1 to 10 nM fMLP (for monocytes) at room temperature. Over an 8-min time course, aliquots of cells were fixed in 3.7% buffered formalin containing 100 μg/ml lysophosphatidylcholine (Sigma Chemical Co., St. Louis, MO), and stained for F-actin according to the method of Howard and Meyer [41]. The cells were stained with NBD-phallacidin at a final concentration of 165 nM for 30–45 min at room temperature, washed twice in fresh HBS containing divalent cations to remove excess unbound stain, and then analyzed by flow cytometry.

Intracellular calcium determination

A 1 mM stock solution of fluo-3/acetoxymethyl ester (fluo-3/AM; Molecular Probes, Eugene, OR) was made up fresh in dry dimethyl sulfoxide (DMSO) containing Phuronic F-127. Serum-pretreated or patients' leukocytes were suspended in calcium-free HBS and exposed to 2 μM fluo-3/AM for 40–50 min at room temperature with gentle agitation. Fluo-3–loaded cells were washed thoroughly in three changes of fresh HBS buffer supplemented with 1 mM CaCl2 to remove excess unbound dye. The cells were filtered through nylon mesh and kept on ice, in the dark, until use in the flow cytometer. Fluorescence intensity was recorded on a linear scale prior to and after the addition of 10 to 100 nM fMLP. To minimize dye loss from cells, samples were kept on ice and allowed to warm to room temperature just before analysis on the flow cytometer [42].

Fluo-3 responds to calcium with an increase in fluorescence intensity, rather than a change in excitation or emission ratio. Therefore, a calibration procedure modified from Tsien et al. [43], Hesketh et al. [44], and Vandenberghe and Ceuppens [45] was employed. For calibration purposes, fluorescence intensity was recorded after the addition of 10 μM nonfluorescent calcium ionophore Br-A23187 in HBS buffer with 2 mM CaCl2 and also after the addition of 2 mM MnCl2. Intracellular calcium concentration was then determined using the following formula:

\[
(Ca^{2+})_{i} = K_d \left( \frac{(F - F_{min})}{(F_{max} - F)} \right)
\]

where \(K_d\) represents the dissociation constant of Ca2+-bound fluo-3, 400 nM; \(F_{max}\) represents the maximum fluorescence recorded after ionophore treatment; \(F\) represents the recorded fluorescence of the experimental samples; and \(F_{min}\) is determined from the MnCl2 recording. The MnCl2–fluo-3 complex is eight times as bright as free fluo-3 [46]; thus the MnCl2 recording multiplied by 0.125 yields \(F_{min}\).

Statistical evaluation of data

Mean channel numbers (MCNs) for each time or treatment were treated as individual parametric values and groups of these values were compared using paired or unpaired t-tests. Probability values < .05 were considered significant.

RESULTS

Chemotaxis assays

A consistent reduction in monocyte and PMN chemotaxis in response to fMLP was seen in cells pretreated with 10% CA serum compared with those pretreated with CT serum. The average reduction in chemotaxis for CA serum–pretreated monocytes was 60% (n = 17) and for PMNs was 24% (n = 17).

CA serum–pretreated leukocytes show less intense myosin staining than CT serum–pretreated leukocytes

Phycoerythrin-conjugated MY-9 staining was used to confirm the location of the monocyte gate. This was necessary because the extraction protocol used before staining for myosin or MLCK resulted in shifted monocyte scatterplots. MY-9 staining of the gated monocyte population revealed that 85% of the cells were monocytes. Because of their low cytoplasmic granularity, the remainder were presumed to be large lymphocytes. The same proportion of monocytes was seen in both CA and CT samples.

Mean channel numbers shown are the averages of three separate experiments (mean ± SD) performed on blood samples from different CT subjects using different CT and CA serum samples for pretreatment (see Table 1). Unstimulated CT serum–pretreated PMNs stained for myosin were seen as discreet unimodal distributions. As a group, unstimulated CT serum–pretreated PMNs had an average MCN of 66 ± 3, whereas unstimulated PMNs pretreated with CA serum had an MCN of 35 ± 3 (P < .01 compared with unstimulated CT). Unstimulated CT serum–pretreated monocytes stained for myosin were seen as broad unimodal or bimodal distributions (Fig. 1) having an average MCN of
TABLE 1. Average Mean Channel Numbers for Normal Leukocytes Pretreated with CT or CA Serum

<table>
<thead>
<tr>
<th>Antimony staining</th>
<th>PMNs</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT serum</td>
<td>CA serum</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>66 ± 3</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>fMLP-treated, 4 min</td>
<td>59 ± 2</td>
<td>52 ± 3</td>
</tr>
</tbody>
</table>

*Results of three separate experiments using serum samples from three different CT subjects and three different CA patients. The MCNs for each group were averaged (mean ± SD).

1 P < .01 compared with unstimulated CT group.

2 P < .01 compared with unstimulated CA group.

62 ± 7, whereas unstimulated monocytes pretreated with serum from CA patients were seen as broad unimodal distributions having an average MCN of 28 ± 8 (P < .01 compared with unstimulated CT).

After fMLP stimulation, myosin staining is increased in CA serum-pretreated but not CT serum-pretreated leukocytes

Upon exposure to 1 nM fMLP for 4 min, the MCN observed for CT serum-pretreated PMNs declined from 66 ± 3 to 59 ± 2 (Table 1). However, under the same conditions the MCN for CA serum-pretreated PMNs increased from 35 ± 3 to 52 ± 3. In monocytes, the MCN observed for CT serum-pretreated monocytes declined from 62 ± 7 to 51 ± 7 after 4 min of exposure to 0.1 nM fMLP. Under the same conditions, the MCN for CA serum-pretreated monocytes increased significantly (P < .01) from 28 ± 8 to 47 ± 8.

MLCK staining was similar for CT and CA serum-pretreated leukocytes

Unstimulated CT serum-pretreated PMNs stained for MLCK (Table 2) were seen as a unimodal distributions having an MCN of 65 ± 9, whereas unstimulated PMNs pretreated with CA serum displayed mean channel numbers of 64 ± 9. Upon exposure to 1 nM fMLP for 4 min, the MCN observed for CT serum-pretreated PMNs was 68 ± 15 and for CA serum-pretreated PMNs was 67 ± 9.

When unstimulated monocytes were pretreated with CT serum and stained for MLCK, a broad unimodal peak was seen; similarly, unstimulated monocytes pretreated with CA serum exhibited unimodal fluorescence peaks. Upon exposure to 0.1 nM fMLP for 4 min, the MCN observed for CT serum-pretreated monocytes was not significantly increased, nor was that for CA serum-pretreated monocytes.

Fluo-3 staining of human neutrophils and monocytes

To determine the baseline level of intracellular calcium, the fluorescence of fluo-3-loaded human PMNs and monocytes was measured by flow cytometry and recorded on the integral scale. Addition of 100 nM fMLP (final concentration) resulted in a severalfold increase in fluorescence that was seen within seconds in both neutrophils and monocytes. This increase in fluorescence was maintained for 10–15 s, whereupon fluorescence began to decrease and returned to near baseline levels within 3 min after stimulus addition.

Pretreatment of leukocytes with human serum (CT or CA did not qualitatively or quantitatively alter the loading of fluo-3, the cellular response to fMLP, or the response to the calibration reagents (ionomycin, Br-A23187, MnCl2). Cells that were pretreated with CA serum showed no differences in fluorescence levels compared to cells pretreated with CT serum. The increase in fluorescence after fMLP stimulation was similar in magnitude, and the gradual return of fluorescence to baseline levels proceeded similarly in both groups.

Table 3 shows that for PMNs pretreated with CT serum or with CA serum, the mean intracellular free calcium levels rose about tenfold following stimulation with formylpeptide. Similarly, in four experiments involving monocytes (Table 4), the intracellular calcium levels for CT serum-pretreated monocytes and for CA serum-pretreated monocytes also rose approximately tenfold upon stimulation with formylpeptide. Thus, the calcium levels for unstimulated and fMLP-treated CA serum-pretreated monocytes and PMNs were not significantly different from those of CT serum-pretreated cells.
TABLE 2. Average Mean Channel Numbers for Normal Leukocytes Pretreated with CT or CA Serum

<table>
<thead>
<tr>
<th>Anti-MLCK staining</th>
<th>PMNs</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT serum</td>
<td>CA serum</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>65 ± 9</td>
<td>64 ± 9</td>
</tr>
<tr>
<td>fMLP-treated, 4 min</td>
<td>68 ± 15</td>
<td>67 ± 9</td>
</tr>
</tbody>
</table>

*Results of three separate experiments using serum samples from three different CT subjects and three different CA patients. The MCNs for each group were averaged (mean ± SD).

1 Not significantly different from CT, not significantly different from unstimulated CA.

NBD-phallacidin staining

Actin polymerization time course

To observe time-related changes in the F-actin content of leukocytes during chemotactic stimulation, leukocytes were exposed to fMLP for times ranging from 0 to 4 min, fixed, stained with NBD-phallacidin, and studied using flow cytometry. Figure 2 (top) represents unstimulated PMNs from healthy control subjects with a baseline MCN of 87 ± 8, which increased to 142 ± 5 after 30 s of fMLP stimulation. The fluorescence MCN declined after 1 min and after 4 min of fMLP stimulation. The MCN of unstimulated CA PMNs in the same experiment was 99 ± 10, but after 30 s of fMLP stimulation the MCN rose to 149 ± 5 and after 4 min the MCN declined to 159 ± 7. The differences in fluorescence between CT and CA PMNs at each time point were not significant.

NBD-phallacidin fluorescence in CT monocytes (Fig. 2, bottom) showed a pattern similar to that seen for PMNs but did not reach a peak until 1 min after exposure to fMLP. In the same experiment, the corresponding CA monocyte sample exhibited the same pattern as the CT monocytes. Again, there were no significant differences in NBD-phallacidin fluorescence between CT and CA monocytes at any time point.

In other experiments, normal leukocytes were pretreated at 37°C for 30 min with either CT or CA serum (Fig. 3), stimulated with fMLP, fixed, and stained with NBD-phallacidin. Control and CA serum-pretreated PMNs (Fig. 3, top) exhibited the rapid rise followed by slow decline in fluorescence described above. Control and CA serum-pretreated monocytes (Fig. 3, bottom) also displayed similar changes in fluorescence on stimulation with fMLP. The MCNs for NBD-phallacidin fluorescence in CT and CA serum-pretreated cells were not significantly different at any of the time points studied.

TABLE 3. PMN Intracellular Calcium

<table>
<thead>
<tr>
<th>Trial</th>
<th>fMLP</th>
<th>+ fMLP</th>
<th>fMLP</th>
<th>+ fMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>128 nM</td>
<td>1071 nM</td>
<td>115 nM</td>
<td>1184 nM</td>
</tr>
<tr>
<td>2</td>
<td>172</td>
<td>1888</td>
<td>90</td>
<td>811</td>
</tr>
<tr>
<td>3</td>
<td>98</td>
<td>1281</td>
<td>128</td>
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<td>113</td>
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<td>126</td>
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<tr>
<td>6</td>
<td>121</td>
<td>1014</td>
<td>108</td>
<td>1083</td>
</tr>
<tr>
<td>7</td>
<td>60</td>
<td>837</td>
<td>137</td>
<td>1675</td>
</tr>
</tbody>
</table>

*CT and CA serum-pretreated cells showed no significant differences in levels of intracellular calcium.

TABLE 4. Monocyte Intracellular Calcium

<table>
<thead>
<tr>
<th>Trial</th>
<th>fMLP</th>
<th>+ fMLP</th>
<th>fMLP</th>
<th>+ fMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>265 nM</td>
<td>1431 nM</td>
<td>1065 nM</td>
<td>1453</td>
</tr>
<tr>
<td>2</td>
<td>134</td>
<td>1492</td>
<td>82</td>
<td>1754</td>
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<td>3</td>
<td>103</td>
<td>1457</td>
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<td>810</td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>903</td>
<td>98</td>
<td>1431</td>
</tr>
</tbody>
</table>

*CT and CA serum-pretreated cells showed no significant differences in levels of intracellular calcium.

Figure 2. NBD-phallacidin staining of human peripheral blood leukocytes isolated from tumor patients or CT subjects. Neutrophils were stimulated with 100 nM fMLP and monocytes were stimulated with 10 nM fMLP at 25°C for the times indicated, then fixed and stained with NBD-phallacidin. Measurements are expressed as the MCN of fluorescence ± SD. Shown is a representative experiment (n = 3). Top, neutrophils; bottom, monocytes.
cells to repetitive stimulation with chemoattractant at any of the time points studied.

DISCUSSION

Myosin, MLCK, actin polymerization, and cytoplasmic free calcium have been quantitated using flow cytometry in unstimulated and formylpeptide-stimulated PMNs and monocytes or in PMNs and monocytes from normal leukocytes pretreated with CT or CA serum. No differences were evident between the CT and CA groups with respect to intracellular free calcium fluxes or actin polymerization in response to chemoattractant stimulation. However, compared with PMNs and monocytes pretreated with CT serum, unstimu-

Fig. 3. NBD-phallacidin staining of human peripheral blood leukocytes pretreated with CT or CA serum. Serum-pretreated cells were exposed to fMLP at concentrations of 100 nM (neutrophils) or 10 nM (monocytes) for the times indicated, then fixed and stained with NBD-phallacidin. Measurements are expressed as the fluorescence MCN ± SD. Shown is a representative experiment (n = 3). Top, neutrophils; bottom, monocytes.

Repellent formylpeptide stimulation

In some experiments, CT and CA serum-pretreated normal human neutrophils and monocytes were stimulated with 10 nM fMLP (PMNs) or 1.0 nM fMLP (monocytes) for times ranging from 0 to 10 min. A rapid increase in MCN was seen after 30 s of fMLP stimulation (Fig. 4), followed by a gradual decline in fluorescence levels as described above. A second aliquot of fMLP at tenfold higher concentrations (100 nM for PMNs and 10 nM for monocytes) was then administered. Both PMNs and monocytes pretreated with CT or CA serum responded with a rise in fluorescence that was evident within 30 s and reached approximately the same maximum MCN that had been recorded with the original fMLP stimulus. A gradual diminution in fluorescence occurred after prolonged chemoattractant stimulation, but after 30 min of stimulation the cells were still to 20 channel numbers above prestimulus levels. No significant differences were noted in the response of CT and CA serum-pretreated
lated and fMLP-stimulated leukocytes pretreated with serum from CA patients exhibited significantly altered levels of myosin staining.

In unstimulated leukocytes pretreated with CA serum, dramatically reduced myosin staining was observed compared with that seen in unstimulated leukocytes pretreated with CT serum. In addition, myosin levels in normal leukocytes pretreated with CT serum decreased markedly, but myosin staining in leukocytes pretreated with CA serum was increased on exposure to fMLP. The level of myosin staining is determined by the amount of myosin associated with the cytoskeleton and the type of fixation/extraction procedure used [18]. Because myosin phosphorylation apparently determines the extent of the association between myosin and the cytoskeleton [15, 47], these findings suggest that myosin phosphorylation or dephosphorylation may be abnormal in CA leukocytes. Wilson et al. [24] have shown, using rat macrophages, that myosin light chain phosphorylation must be regulated within narrow limits to promote optimal cell locomotion. When myosin phosphorylation is abnormally increased or decreased, motility is reduced. If myosin phosphorylation is altered in CA leukocytes, this alone could account for the inhibition of cell polarization [48, 49] and chemotaxis seen in cells from CA patients.

Dharmawardhane et al. [18] have shown that the myosin II content of the Dictyostelium cytoskeleton peaks 25–30 s after chemoattractant stimulation and reaches a minimum, about 50% below baseline levels, 40 s after stimulation. Levels of cytoskeleton-associated myosin remained significantly below baseline throughout the remainder of the 70-s time course studied there. The myosin content of human leukocytes pretreated with CT serum seen here after 1 min (data not shown) or 4 min of fMLP stimulation was correspondingly reduced. MLCK staining in leukocytes pretreated with CT or CA serum was similar before and after fMLP stimulation. This suggests that the markedly decreased myosin staining observed after fMLP stimulation was not simply the result of increased nonspecific protein extraction. Thus, the decrease in myosin staining seen in unstimulated PMNs and monocytes pretreated with CA serum appears to be selective for myosin.

Chemoattractant stimulation of leukocytes results in a dramatic, transient increase in intracellular free calcium levels similar to that reported elsewhere [42, 50, 51]. Baseline fluorescence levels and fMLP-stimulated fluorescence in unstimulated neutrophils and monocytes were not significantly different for CT and CA serum-pretreated cells (Tables 3 and 4). This indicates that the ability of CA serum-pretreated cells to mobilize intracellular free calcium in response to chemoattractant stimulation was not altered. It is thought that a serum-borne cell-directed inhibitor may be responsible for the chemotactic defect seen in CA patients' monocytes [52, 53]. Low-molecular-weight extracts prepared from type C retroviruses (Friend leukemia, Moloney, Rauscher) are potent inhibitors of macrophage chemotaxis in mice [54]. A protein immunologically indistinguishable from the retroviral envelope protein p15E is found at high levels in extracts prepared from several types of murine and human tumor cells [55]. The peptide LDLLFL, a p15E homologue, inhibits intracellular free calcium fluxes in monocytes as seen with indo-1 in response to stimulation with fMLP but not with C5a or leukotriene B4 [56]. In contrast, the present study shows no such inhibition of calcium fluxes in CA serum-pretreated PMNs or monocytes.

Important differences between the study of Oostendorp et al. [56] and the present study may account for this. First, whole serum from CA patients was used here, whereas Oostendorp et al. used purified hexapeptide. Whole serum consists of a complex mixture of normal and tumor-associated factors. These factors may affect a variety of leukocyte function, but the consistent and overriding effect is that of leukocyte chemotactic inhibition. On the other hand, LDLLFL is an analogue of a small portion of the retroviral envelope protein p15E, which is putatively related to the chemotaxis inhibitor present in CA patients' serum. This peptide may interact with leukocytes in a manner quite different from that of the authentic cancer-related chemotactic inhibitor. Indeed, the ability of such synthetic p15E-related peptides to inhibit leukocyte chemotaxis is questionable. Second, the LDLLFL hexapeptide was applied during the entire time course of formylpeptide stimulation but leukocytes were exposed to CA serum before, not during, formylpeptide stimulation. Under these conditions, the LDLLFL hexapeptide may compete directly with fMLP for binding to the formylpeptide receptor and thereby affect subsequent stages of cell activation, including intracellular free calcium fluxes. However, using the protocol employed here, competitive cell surface binding effects are minimized, persistent chemotactic inhibition is still observed, and relevant aspects of cell activation can be studied to advantage.

Actin polymerization and remodeling of the actin cytoskeleton during chemotaxis may occur independent of changes in intracellular free calcium. Consistent with other published reports [3, 41], flow cytometric analysis of PMNs and monocytes stained with NBD-phallacidin showed fluorescence rising within 10 s of exposure to fMLP, peaking at 30 s, and then gradually diminishing toward baseline levels. No differences were evident between either PMNs or monocytes pretreated with CT or CA serum with respect to actin polymerization in response to chemoattractant stimulation. In addition to their initial response to chemoattractant challenge, leukocytes must continuously or repeatedly respond to ongoing or changing stimulation by chemoattractants [3]. Therefore, the effect of repeated fMLP exposure on actin polymerization was compared in CT and CA serum-pretreated neutrophils and monocytes. Both groups showed similar changes in NBD-phallacidin staining in response to a second pulse of fMLP at a tenfold higher concentration than the initial stimulus. This further indicates that actin polymerization and depolymerization were not affected in CA serum-pretreated leukocytes.

In summary, serum-borne p15E-related inhibitors may be responsible for the chemotactic defect seen in CA patients' monocytes. This inhibitor or its synthetic peptide analogues cause decreased monocyte polarization in response to chemoattractant [48, 49], alterations in formylpeptide receptor expression [56, 57], suppression of the respiratory burst [58], inhibition of protein kinase C-related cell functions [59], and inhibition of a range of other responses in human leukocytes. This study has shown that CA serum affects neither intracellular calcium fluxes nor actin polymerization in resting or chemoattractant-activated leukocytes. However, myosin staining was vastly different in CA and CT serum-pretreated leukocytes. This alteration in CA serum-pretreated leukocytes may contribute to the defective leukocyte polarization and chemotaxis observed in serum-pretreated leukocytes and in leukocytes from patients with advanced cancer. Inhibition of leukocyte function as a result of impaired locomotion may contribute to the inability of these immune effector cells to deter the growth and spread of neoplasia and to the susceptibility of cancer patients to life-threatening bacterial infections [60].
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REFERENCES


