The Ionic Basis of Chemotaxis

Separate Cation Requirements for Neutrophil Orientation and Locomotion in a Gradient of Chemotactic Peptide

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The behavior of cells undergoing chemotaxis may be analyzed in terms of their orientation, a static characteristic, and of their locomotion. We have examined the extracellular divalent cation requirements for orientation and locomotion of rabbit polymorphonuclear leukocytes (neutrophils) in a gradient of the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe) using the chemotaxis chamber recently developed by Zigmond.[11] This chamber allows direct observation of cells attached to glass coverslips as they move up a gradient of chemotactic agent established across a 1-mm bridge. The orientation of neutrophils in the direction of the gradient was equally efficient whether cells and F-Met-Leu-Phe were suspended in medium supplemented with both Ca^{2+} and Mg^{2+} (complete medium), with Mg^{2+} but not Ca^{2+} (by simple omission of Ca^{2+} or by addition of EGTA), or with nonsupplemented medium (by omission of Ca^{2+} and Mg^{2+} or by addition of EDTA). These data confirm and extend Zigmond's earlier observation that exogenous divalent cations are not required for polymorphonuclear leukocyte orientation toward the chemotactic peptide. In contrast, cell locomotion, determined by linking the chemotaxis chamber to a time-lapse videocassette recorder and TV monitor, is markedly affected by the medium's content of divalent cations. Cells suspended in medium supplemented with Mg^{2+} but not calcium (by omission or chelation) or in nonsupplemented medium moved on the average 25% more rapidly than cells in complete Ca^{2+} and Mg^{2+} medium. Although the simple omission of Mg^{2+} does not prevent chemotaxis, chelation of Mg^{2+} in the medium completely abolishes leukocyte locomotion. Addition of varying concentrations of Mg^{2+} to the buffer in the presence of EDTA established that cell movement is fully restored by Mg^{2+} concentrations in the range of 3×10^{-9} M, concentrations easily attained in the absence of added Mg^{2+}. It was concluded that neither Ca^{2+} nor Mg^{2+} is needed for orientation in response to F-Met-Leu-Phe. However, low levels of exogenous Mg^{2+} but not Ca^{2+} are required for effective locomotion of neutrophils in the Zigmond chamber. This result contrasts with data obtained in the Boyden chamber, where exogenous Ca^{2+} is considered essential for maximum chemotactic response. (Am J Pathol 1980, 98:749-768)

THE DIRECTED MOVEMENT of polymorphonuclear leukocytes (neutrophils, PMNs) into or through the pores of a filter towards a chemotactic factor contained in a Boyden chamber has been thought to require

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exogenous cations, particularly Ca^{2+}. For example, Becker and Showell,[1] Gallin and Rosenthal,[2] Wilkinson,[3] and Estensen et al [4] have demonstrated that both Ca^{2+} and Mg^{2+} are required for maximum PMN migration in a gradient of a bacterial peptide or the C5a fragment of complement generated by endotoxin; Boucek and Snyderman [5] have reported inhibition of chemotaxis towards endotoxin-activated human serum when lanthanum was present to inhibit Ca^{2+} transport; and Gallin et al [6] have described the specific deposition of pyroantimonate, presumed to mark local concentrations of cations, at the leading surface of PMNs oriented in filters towards a gradient of endotoxin-activated serum. In separate experiments, it has been shown that the chemoactic peptide N-formylmethionyl-leucylphenyl-alanine (F-Met-Leu-Phe) as well as other chemotactic agents, ie, C5a, may cause a transient membrane hyperpolarization [7,8] and may accelerate the transport of Na^+, K^+ and Ca^{2+} across the membranes of PMNs in cell suspensions.[7] Oliver et al,[10] using the chemotaxis chamber developed by Zigmond,[11] in which cell orientation is observed directly through a phase microscope, have demonstrated that recruitment of actin to the anterior edge of PMNs is associated with their orientation in a gradient of F-Met-Leu-Phe. By analogy to muscle systems where actomyosin contraction requires the release of Ca^{2+}, it could be argued that Ca^{2+} influx may follow peptide binding and promote this specific redistribution of leukocyte actin.

In contrast to these data implicating Ca^{2+} in PMN locomotion stimulated by chemotactic factors, Zigmond [11] reported that orientation of monolayers of PMNs on glass coverslips in a gradient of F-Met-Leu-Phe does not require exogenous divalent cations. This discrepancy stimulated the present systematic study of divalent cation requirements for orientation and movement of PMNs in a gradient of chemotactic peptide.

We have confirmed Zigmond's report that orientation of PMNs on glass is independent of exogenous divalent cations. As we will show, this cation independence holds over a range of incubation times and concentrations of F-Met-Leu-Phe. We have also extended the analysis to cell locomotion. The extension was made possible by coupling a time-lapse video display to the light-microscopic system devised by Zigmond to observe cell orientation. We can thus observe and simultaneously record the motile behavior of a population of responding cells. We report that exogenous Ca^{2+} is not required for, and indeed may decrease, the directed movement of rabbit PMNs across a glass coverslip towards a gradient of F-Met-Leu-Phe. Exogenous Mg^{2+}, on the other hand, must be present for cell locomotion to occur.

Materials and Methods

Cells

Female New Zealand rabbits (2–3 kg) were given injections intraperitoneally of 200–400 ml of sterilized isotonic saline containing 0.1% glycogen. The peritoneal leukocytes were removed 12–15 hours later as previously described.[12] Differential counts indicated that the exudates contained approximately 85% polymorphonuclear leukocytes and 15% monocytes.

Buffers

Exudate suspension (4–5 ml) was pipetted into centrifuge tubes containing Ca^{2+} and Mg^{2+}-free Hanks' medium supplemented with appropriate cations and/or chelating agents. The mediums used were Hanks' + 0.7mM Mg^{2+} + 1.6 mM Ca^{2+} (complete medium); Hanks' + 0.7 mM Mg^{2+}; Hanks' + 0.7 mM Mg^{2+} + 2.0 mM EGTA (Ca^{2+}-depleted Hanks'); Hanks' + 2 mM EDTA (Mg^{2+} + Ca^{2+}-depleted Hanks'); and nonsupplemented Hanks'. In all cases cells were centrifuged twice, and the cell pellets were resuspended in the same medium containing 1 mg/ml crystalline bovine serum albumin (BSA). Cell concentrations were adjusted to 3×10^6 cells/ml in all experiments. Cells were kept on ice until ready for use.

Chemotaxis

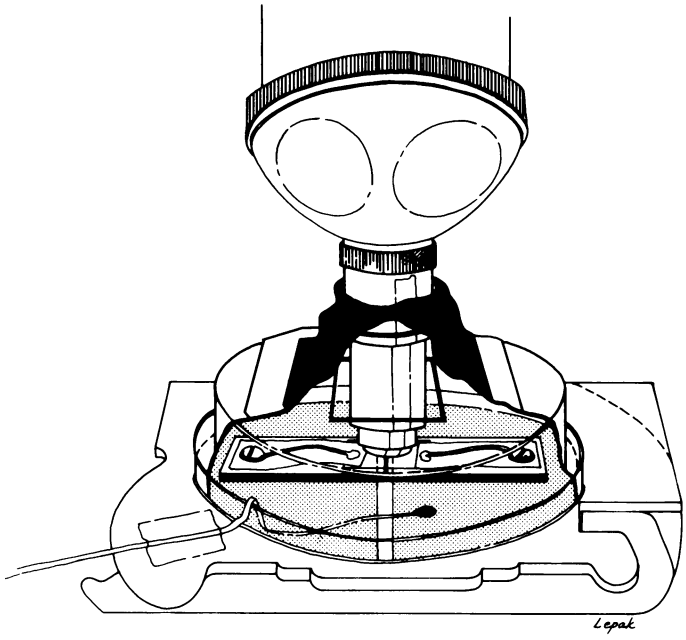
The chemotaxis chambers were set up essentially as described by Zigmond.[11] In brief, 25 μl of the cell suspension were pipetted from edge to edge in a narrow bead along the center of a 22 × 22 mm No. 1 glass coverslip. The cells attached to the glass surface to form a monolayer during incubation for approximately 15 minutes in the 37 C humidity chamber designed by Hawkins and Berlin.[13] The number of glass-adherent cells was the same for PMNs plated in all four mediums. The coverslip with adherent PMNs was rinsed twice through beakers containing the same buffer at room temperature, then inverted and lowered onto a Plexiglas slide with two wells separated by a bridge 1 mm in diameter. The cell monolayer was centered on the bridge, the coverslip secured in place with brass clips, excess buffer was removed by gentle suction, and the wells were filled with the appropriate solutions. Except where noted, peptide solution (100 μl) occupied one well, while the second well contained buffer only.

For orientation studies these chemotaxis chambers were usually incubated for 30 minutes at 37 C in the humidity chamber, then transferred to the microscope stage and immediately photographed.

For motility studies, a similar preincubation was often employed. However, chemotaxis chambers were subsequently transferred into a 100-mm Petri dish that was lined with a damp towel to maintain humidity. The dish was set in a Plexiglas frame designed to fit on the stage of a Zeiss Photomicroscope III and to be moved by use of the stage control knobs. The complete apparatus is illustrated in Text-figure 1. A flexible nosepiece attaches the Petri dish humidity chamber to the objective. The chemotaxis chamber can be seen inside the humidity chamber. Stage temperature was maintained at 37 C by use of a Sage model 279 air curtain incubator (Orion Instruments, Cambridge, Mass). Temperature was constantly monitored in the chamber by use of a thermocouple (Telethermometer, Yellow Springs Instrument Co., Yellow Springs, Ohio). Use of this chamber allowed prolonged observation of cells without fluctuation of temperature or loss of medium by evaporation.

Orientation Studies: Quantitation

For orientation studies, cells were viewed through a Zeiss photomicroscope using a 16× phase contrast objective. Only cells that were in the center 1 cm of the bridge length and



TEXT-FIGURE 1—The humidity and chemotaxis chambers as they appear on the microscope stage. The complete apparatus is set in a Perspex frame tooled to fit the contours of the microscope stage and thus allow positioning of the apparatus with the stage control knobs.

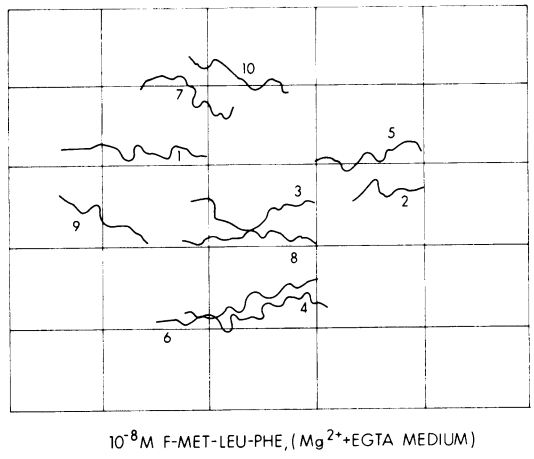
center 400 μ of the bridge width were recorded. This eliminated any variability due to the edge effects noted by Zigmond. Cells were photographed using Kodak Tri-X-film and on occasion Kodak SO-115 film. In each experiment, the bridge was scanned and two fields were chosen at random for photography. Duplicate slides were recorded at each dilution.

The resulting photographs contained two major groups of cells. In the absence of peptide, most cells were round or irregularly shaped and were scored as nonresponding PMNs. In the presence of peptide most cells developed an elongated shape with a distinct anterior lamellipodium and a uropod that was directed away from the peptide gradient. These were scored as oriented or responding cells provided that the angle subtended by the long axis from lamellipodium to uropod deviated no more than 80 degrees from the direction of the gradient. The percentage of orientation was determined by dividing this number by the total number of PMNs in the field. The final value was corrected for the percentage of oriented cells in chambers containing buffer only. No more than 5% of the cells were oriented in the absence of a gradient of peptide. Macrophages, which usually comprised 10–15% of the monolayer, were omitted from the counting procedure.

The broad angle of orientation was chosen in order to include cells that developed an elongated shape but imperfect orientation at low (10^{-10}–10^{-11} M) concentrations of peptide. However, calculation from photographs of cells oriented for 30 minutes in an optimal peptide gradient (10^{-8} M F-Met-Leu-Phe) showed that, in fact, 47% of these cells deviated by only 10 degrees or less from the direction of the gradient and 93% of oriented cells lay within 40 degrees of the direction of the gradient. Thus, PMNs are capable of a highly accurate directional response to a gradient of F-Met-Leu-Phe (see Figure 1).

Kinetic Studies: Quantitation

For the kinetic experiments, a Newvicon television camera (DAGE-MTI, Michigan City, Ind) was mounted on the microscope in series with a time-lapse videocassette recorder (NEC, Elk Grove, Illinois) and a 14-inch video monitor (Setchell Carlson, New Brighton, Maine). In general, the chambers were incubated with the desired gradient for



TEXT-FIGURE 2—PMN locomotion in a gradient of 10^{-8} M F-Met-Leu-Phe. A typical experimental tracing is shown in which the paths of 10 cells incubated in medium with Mg^{2+} and EGTA are followed from their entry into one of the central 9 rectangles of a stencil taped over the video screen until their exit from the chosen square. The width of each rectangle corresponds to 69.0 μ in the microscope. The reservoir with the F-Met-Leu-Phe is to the left of the figure, and the cells are migrating from right to left.

20 minutes at 37 C, and then time-lapse photography was started at a 9:1 time ratio for 30 minutes. Duplicate chambers were used for each condition. The video screen used to view cells was calibrated so that each centimeter of screen equaled 13.6 μ. For the experiments reported here, kinetic data were recorded by tracing the paths of individual cells on a clear stencil divided into 9 squares, each 2 × 1½ inches. This stencil was firmly taped across the center of the video screen. A typical example is shown in Text-figure 2. A random number table was used to pick which square would be recorded on each run. A stopwatch was activated when the first cell moved into that square. The path of the cell across the square and into the next square was traced with a fine-pointed wax pencil. The elapsed time for movement was recorded and the distances traveled measured by rolling a map measurer calibrated in centimeters along the pencil tracing. We routinely determined the percentage of responding cells in each field at the end of the experiment by dividing the number of cells that had moved from a square by the number of cells present initially times 100.

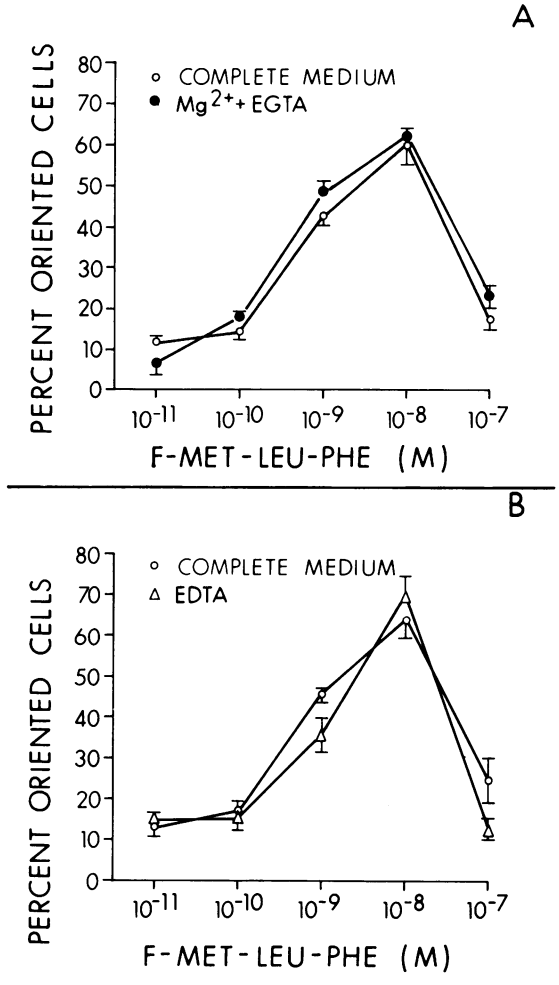
Determination of Magnesium

We determined levels of Mg^{2+} in Hanks' medium by atomic absorption spectrophotometry at 2852 Å (magnesium line), using a Perkin-Elmer Model 107 spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn) with an air-acetylene flame. Magnesium standards and reagent blanks were run with each batch of samples. For determination of intracellular magnesium levels, cells were washed 3 times in nonsupplemented Hanks' medium or medium containing EDTA and resuspended at 2×10^7 cells/ml. Diluted whole cell suspensions or supernatants of triton-treated cells (0.1% final concentration) were then analyzed.

Results

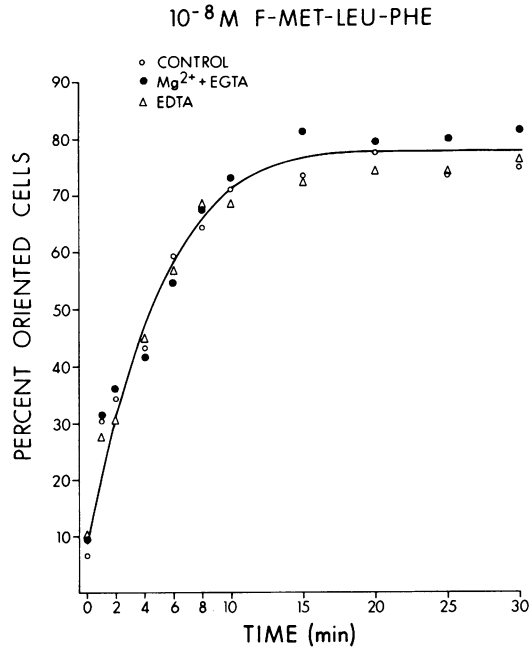
PMN Orientation in a Gradient of F-Met-Leu-Phe

In initial experiments, PMNs were simply incubated for 30 minutes in various gradients of F-Met-Leu-Phe, and the percentage of oriented cells was determined. The resulting dose-response curves for cell orientation are shown in Text-figure 3.



TEXT-FIGURE 3—Dependence on peptide and cation concentration of PMN orientation towards F-Met-Leu-Phe. Cells and peptide were prepared in medium supplemented with Ca^{2+} and Mg^{2+} (*open circles*), with Mg^{2+} + EGTA (*solid circles*), and with EDTA (*open triangles*). Coverslips with attached cells were inverted onto chemotaxis chambers and exposed at 37 C for 30 minutes to the indicated gradients of F-Met-Leu-Phe. Cell orientation was subsequently recorded by photography of random fields. The percentage of oriented cells was calculated as described in Materials and Methods.

For cells in complete Hanks' medium, maximum orientation (approximately 70%) was found at 10^{-8} M F-Met-Leu-Phe (Text-figure 3A, B, open circles). With 10^{-9} M and lower concentrations of F-Met-Leu-Phe, we noted an overall decrease in orientation. At 10^{-7} M F-Met-Leu-Phe, we observed oriented cells only near the buffer well and not in the center of the bridge; this failure of orientation at high peptide concentration probably reflects saturation of the F-Met-Leu-Phe receptors, followed by their deactivation over the whole membrane. Similar data (not shown) were obtained when PMNs were exposed to gradients of F-Met-Leu-Phe run against a 10-fold dilution of F-Met-Leu-Phe rather than against buffer alone.



TEXT-FIGURE 4—Time course of PMN orientation in a gradient of 10^{-8} M F-Met-Leu-Phe. Chemotaxis chambers were filled with peptide, then immediately placed on the microscope stage and recorded by photography at short (2 minutes or less) intervals over 35 minutes of incubation. Data are the average of 2 complete experiments, each run in duplicate.

The Role of Cations in PMN Orientation

The role of extracellular Ca^{2+} and Mg^{2+} in the orientation of PMNs to F-Met-Leu-Phe was determined from dose-response curves generated after a 30-minute incubation in medium supplemented with either Mg^{2+} and EGTA (Ca^{2+}-depleted medium) or EDTA alone (to deplete both Ca^{2+} and Mg^{2+}). The resulting dose-response curves are identical, whether cells and peptide are in complete medium (Text-figure 3A, B, open circles), in medium containing Mg^{2+} and EGTA (Text-figure 3A, closed circles), or in EDTA (Text-figure 3B, open triangles). Clearly, neither Ca^{2+} nor Mg^{2+} is needed in the medium for rabbit PMNs to respond to the chemotactic factor. This finding is consistent with Zigmond's earlier observation that orientation of human PMNs towards F-Met-Leu-Phe (10^{-8} M) is unaffected by the presence of EDTA or EGTA. Our data show that this absence of cation dependence for orientation extends over the entire dose-response range of F-Met-Leu-Phe.

The time and extracellular cation requirements for PMN orientation in response to 10^{-8} M peptide were further investigated by incubation of chemotaxis chambers directly in the humidity chamber on the microscope stage. Still photographs were taken every minute for 35 minutes, and the percentage of orientation of the cells was determined. The results are shown in Text-figure 4. Approximately 30% of the cells were oriented

within 2 minutes after establishing the gradient, and maximum orientation (around 70%) was achieved within 15 minutes. This high degree of orientation was subsequently maintained for at least another 30 minutes. The rates of orientation and the percentage of oriented cells were identical in all of the mediums tested. This further established that the orientation response is not dependent on the presence of extracellular Ca^{2+} and Mg^{2+}.

However, an interesting difference emerged when mean uropod length was calculated in a typical set of photographs. Uropod length was estimated from prints by rolling the calibrated map measurer along the total length (from leading edge to end of uropod) of cells incubated with F-Met-Leu-Phe and then subtracting the average diameter of cells incubated with buffer alone. At 10^{-8} M F-Met-Leu-Phe, the mean length determined from the photographs of cells in complete medium and in medium containing Mg^{2+} + EGTA was 39 ± 2 (N = 137) and 42 ± 2 μ (N = 252), respectively. In contrast, cells in medium with EDTA showed a significantly reduced mean uropod length of 19 ± 2 μ (N = 242).

PMN Locomotion: General Observations

PMNs incubated with buffer showed only minimal directional movement, although occasional cells crawled in a random manner. The typical rounded appearance of these immobile cells is illustrated in the top frames of Figure 1, where cells were photographed 0.5 minutes after introduction of F-Met-Leu-Phe (at the immediate onset of the cellular response).

In contrast, the majority of cells incubated for 10 minutes or longer in either complete or Ca^{2+}-depleted medium in a gradient of 10^{-8} M F-Met-Leu-Phe showed directional movement towards the chemotactic peptide. This movement appeared to involve extension and forward attachment of a lamellipodium, followed by contraction of the cytoplasm toward the leading front. Eventual dislodging and shrinking of the substrate-adherent uropod occurred, and the process was repeated in a directional manner. Some cells, however, could pull the entire cell mass forward without apparent resistance from the uropod, and a small proportion of moving cells (around 15%) actually failed to develop a uropod. These latter cells were notable because of their high rate of movement, compared with cells with adherent uropods; they could on occasion be seen moving at speeds greater than 25 μ/min. Similar PMN shapes and modes of locomotion have been described at length by Ramsey,[14,15] Bessis,[16] and others for both randomly moving cells and cells moving toward particulates such as clumps of bacteria or laser-irradiated erythrocytes.

Cell orientation preceded forward movement. Thus, in Figure 1A, partial orientation is apparent after 5 minutes, and most PMNs are oriented by 10 minutes. However, the same cells occupy rather similar positions between 0.5 and 10 minutes of incubation, and marked displacement is not seen until 15 minutes. Motility measurements (below) were thus initiated after a 15–20 minutes incubation to ensure that full motile activity was expressed during the assay period.

At the optimal concentration of peptide (10^{-8} M F-Met-Leu-Phe), 85–90% of the cells were routinely found to be moving after a 20-minute period of incubation. This value is higher than values obtained for the percentage of oriented cells in gradients of 10^{-8} M F-Met-Leu-Phe (usually around 70%) (Text-figures 3 and 4) and points to an interesting paradox: the orientation assay scores cells with prominent uropods (often the slower moving cells) and may exclude some of the most rapidly moving PMNs.

PMN Locomotion: Quantitative Aspects

Preliminary studies in Mg^{2+}-containing medium showed that gradients of 10^{-8} M F-Met-Leu-Phe led to the most rapid movement as well as the most accurate orientation of PMNs. At submaximal doses of F-Met-Leu-Phe, we noted a reduced percentage of responding cells (consistent with the reduced percentage of oriented cells shown in Text-figure 3), a greater variability in the angle of orientation (discussed above), and an apparent reduction in the rate of movement. For example, at 10^{-8} M F-Met-Leu-Phe, a population of PMNs in complete medium showed 85% to be moving cells and an average velocity of 10 μ/min, while at 10^{-10} M F-Met-Leu-Phe only 15% of PMNs from the same cell population showed directed movement, and the average velocity was less than 5 μ/min.

Preliminary analyses also showed that replicate populations of PMNs incubated in complete medium with an optimal peptide gradient (10^{-8} M F-Met-Leu-Phe; 20–50 minutes after establishing the gradient) developed similar but not identical rates of movement (Table 1, Experiments 1–3). In contrast, duplicate measurements on the same batch of cells always gave extremely close estimates of cell motility (Table 1, Experiment 3). Hence, all analyses of cation effects on absolute rate were made within experiments and data were simply compared among experiments.

Cation Requirements for PMN Locomotion

Initial qualitative experiments revealed a striking dependence of locomotion on Mg^{2+}. Thus, with 10^{-8} M F-Met-Leu-Phe, cells in both complete or Ca^{2+}-depleted (Mg^{2+} alone or Mg^{2+} + EGTA) medium could be seen moving in the direction of the gradient. This is clearly illustrated in

Table 1—The Role of Ca^{2+} in PMN Locomotion

	Rate of PMN movement (μ/min ± SEM)*		
	Hanks' + Ca^{2+} + Mg^{2+}	Hanks' + Mg^{2+}	Hanks' + Mg^{2+} + EGTA
Experiment 1†	8.28 ± 1.06 (10)‡§	12.42 ± 1.54 (10)	14.61 ± 1.53 (15)
Experiment 2	8.21 ± 0.66 (10)§	16.17 ± 0.88 (10)	14.20 ± 0.95 (11)
Experiment 3A	11.05 ± 1.45 (10) ‖	15.59 ± 1.72 (10)	14.69 ± 0.86 (10)
Experiment 3B	10.34 ± 1.16 (10) ‖	16.90 ± 0.94 (10)	15.98 ± 0.94 (10)

* Fifteen-minute preincubation in a gradient of 10^{-8} F-Met-Leu-Phe versus buffer.

† First column results significantly different at $P < 0.05$ from results of second and third columns (one-way analysis of variance).

‡ Number of cells measured per chamber.

§ In Experiments 1 and 2 the cells analyzed (number in parentheses) were selected at random from two tapes obtained by setting up delicate chambers.

‖ The duplicate chambers (A and B) were analyzed separately.

the successive frames of Figure 1A. Cells in EDTA-containing medium, thus depleted of both Ca^{2+} and Mg^{2+}, were also oriented in the direction of the gradient (Text-figures 3 and 4). However, these cells were completely unable to locomote (Figure 1B). Instead, rhythmic and repeated attempts to detach the uropods were observed over long periods of incubation, with no resulting directional movement. This local but not translatory movement is readily seen by tracing the marked cells in Figure 1B through 30 minutes of incubation.

The requirement of Mg^{2+} for effective locomotion on glass coverslips was confirmed by replacement experiments. A front was created on a monolayer prepared in EDTA medium by scraping through the middle of the cell monolayer with a microscope slide. The coverslip was then washed and positioned on the bridge such that the cell front was in the center of the bridge, and a gradient of 10^{-8} M F-Met-Leu-Phe in EDTA buffer was run for 30 minutes. The cells were fully oriented but showed no locomotion. The wells were then emptied by gentle suction, medium containing Mg^{2+} + EDTA was added in the same gradient concentration, and direction and time-lapse photography was begun. Within 30 seconds after the replacing of Mg^{2+} in the medium, the cell front began to move across the screen.

No effect of Ca^{2+} on locomotion could be established by qualitative observation. However, a reproducible effect of Ca^{2+} on motility became evident when the behavior of individual cells was analyzed for the rate and distance of their movement as recorded on time-lapse videotape. As shown in Table 1, cells incubated in complete medium moved toward 10^{-8} M F-Met-Leu-Phe at between 8 and 11 (average = 10) μ/min,

Table 2—The Effect of Increased Mg^{2+} * on PMN Locomotion

	Rate of movement (μ/min ± SEM)	
	Hanks' + .7 mM Mg^{2+}	Hanks' + 2.3 mM Mg^{2+}
Experiment 1A†	14.43 ± 0.56 (10)‡	13.61 ± 0.77 (10)
Experiment 1B	15.06 ± 1.53 (10)	14.60 ± 0.70 (10)
Experiment 2A†	15.87 ± 0.75 (10)	16.10 ± 1.14 (10)
Experiment 2B	15.60 ± 0.68 (10)	15.76 ± 1.38 (10)

* Fifteen-minute preincubation in a gradient of 10^{-8} F-Met-Leu-Phe versus buffer.

† Results of first column not significantly different at $P < .05$ (Student t test) from results of second column.

‡ Number of cells measured per chamber.

whereas cells in medium containing Mg^{2+} alone or Mg^{2+} + EGTA moved at between 12 and 17(average = 15) μ/min. This substantial increase in cell velocity caused by omission or chelation of Ca^{2+} could also be seen in time-lapse traces recorded with 10^{-9} M F-Met-Leu-Phe (data not shown). The results in Table 2 indicate that this apparent decreased activity in the presence of Ca^{2+} could not be explained simply by the additive effect of concentrations of 0.7 mM Mg^{2+} and 1.6 mM Ca^{2+}, since cells tested in the presence of 2.3 mM Mg^{2+}, the total divalent cation concentration in complete medium, showed no decreased activity.

Magnesium Dependence of Chemotaxis: Further Analysis

In an attempt to establish the minimal essential Mg^{2+} requirement for locomotion, chemotaxis was measured for cells incubated in nonsupplemented Hanks' medium after being washed in the same or in EDTA-supplemented Hanks' medium. As shown in Table 3, the EDTA wash did not inhibit locomotion; furthermore, cells were able to move in the absence of added Mg^{2+}. These results suggest that if Mg^{2+} is indeed required for locomotion, as indicated by the failure of cell movement in Hanks' medium containing EDTA, then either the buffer is contaminated with enough Mg^{2+} or cells can release sufficient Mg^{2+} into the medium to support this movement.

Measurement of the concentration of Mg^{2+} indicated that contaminating levels of Mg^{2+} in nonsupplemented Hanks' medium were about 1–5 μM. In order to determine if this represents a sufficient Mg^{2+} concentration to support locomotion, it was necessary to have means of providing the cells with a concentration of Mg^{2+} less than the contaminating concentration. To attain this low concentration, it was necessary to measure cell motility in the presence of 1 mM EDTA to which

Table 3—The Effect of EDTA* on PMN Locomotion

	Hanks' buffer minus Ca^{2+} and Mg^{2+}	Washed 2 times in Hanks' buffer containing 2 mM EDTA and tested in Hanks' buffer without the added Ca^{2+} and Mg^{2+}	Hanks' buffer without added Ca^{2+} and Mg^{2+} but with 2 mM EDTA
Experiment 1A	21.71 ± 0.90† (10)§	21.31 ± 0.84 (10)	No locomotion
Experiment 1B	18.93 ± 0.57 (10)	16.41 ± 0.83 (10)	No locomotion
Experiment 2A	16.91 ± 0.57 (10)	15.70 ± 0.86 (10)	No locomotion
Experiment 2B	14.31 ± 0.69 (10)	14.32 ± 0.74 (10)	No locomotion

* Fifteen-minute preincubation in a gradient of 10^{-8} F-Met-Leu-Phe versus buffer.
† Results in first column not significantly different at $P < .05$ (Student t test) from those of second column.
‡ Rate of PMN movement (μ/min ± SEM).
§ Number of cells measured per chamber.

varying concentrations of Mg^{2+} were added. The free Mg^{2+} concentration was calculated utilizing the K_d for Mg^{2+} EDTA of 2×10^{-9} M.[23] The results of two experiments are shown in Table 4: the data indicate that at 1×10^{-9} M free Mg^{2+} locomotion is abolished, whereas as little as 3×10^{-9} M Mg^{2+} appears to support locomotion. We emphasize that these are relative values and may in fact be up to an order of magnitude too low, since the stability constants for EDTA are generally measured between 25 and 30 degrees. Even so, micromolar levels of Mg^{2+} are adequate to support locomotion.

Table 4—Titration of the Mg^{2+} Concentration Dependence of PMN Locomotion*

	Rate of PMN movement (μ/min ± SEM)		
	Hanks'† + .7mM Mg^{2+}	Hanks' + 3×10^{-9} M Mg^{2+}‡	Hanks' + 1×10^{-9} M Mg^{2+}‡
Experiment 1A	22.27 ± 0.96 (10)§	23.67 ± 1.14 ‖ (10)	No locomotion
Experiment 1B	21.57 ± 1.62 (10)	20.27 ± 0.92 (10)	No locomotion
Experiment 2A	23.65 ± 0.94 (10)	22.32 ± 0.94 (10)	No locomotion
Experiment 2B	18.23 ± 1.32 (10)	19.77 ± 1.19 (10)	No locomotion

* Fifteen-minute preincubation in a gradient of 10^{-8} F-Met-Leu-Phe versus buffer.
† In all instances, 1 mM EDTA with no Ca^{2+} but added Mg^{2+}.
‡ Calculated effective concentration of Mg^{2+}; see text.
§ Number of cells measured per chamber.
‖ Results in first column not significantly different at $P < 0.05$ (Student t test) from those of second column.

Table 5—Magnesium Efflux From Polymorphonuclear Leukocytes (PMN)*

FMLP†	Time (min)	Experiment 1 μM	Experiment 2 μM	Experiment 3 μM
0	5	1.83 ± 0.22‡	4.76 ± 0.06	4.36 ± 0.18
	10	2.76 ± 0.16	5.76 ± 0.23	4.75 ± 0.20
	15	3.29 ± 0.28	6.88 ± 0.23	5.39 ± 0.64
10^{-6} M	5	2.2 ± 0.08	4.36 ± 0.08	4.24 ± 0.10
	10	2.98 ± 0.06	4.83 ± 0.40	4.58 ± 0.26
	15	4.12 ± 0.24	7.02 ± 1.05	5.56 ± 0.24

* PMNs (2×10^7/ml) were incubated at 37C in Hanks' medium without added Mg^{2+} for the times indicated. The cells were then pelleted, and the Mg^{2+} concentration was measured in the incubation media.

† Formylmethionyl-leucyl-phenylalanine (F-Met-Leu-Phe).

‡ Increase in extracellular Mg^{2+} concentration (μM) over buffer controls (buffer controls respectively 2.1, 1.8, 2.04 μM Mg^{2+} in the three experiments.

Further measurements showed that rabbit PMNs contain substantial levels of free Mg^{2+}. With a value of 4×10^{-10} cu cm for cell volume and 4.3×10^{-8} mg protein/cell,[17] the concentration of intracellular Mg^{2+} in the rabbit neutrophil was calculated to be $2.02 \pm .55 \times 10^{-3}$ μg Mg^{2+}/μg protein (8.7 ± .47 mM), a level significantly higher than in the physiologic buffer (0.7 mM) used in our studies. We therefore considered the possibility that neutrophils, suspended to our usual concentration (3×10^{-6} PMNs/ml) in Hanks' buffer without Mg^{2+} or Ca^{2+}, may supplement the extracellular cation levels by releasing Mg^{2+}. As shown in Table 5, a time-dependent increase in Mg^{2+} concentration occurred in the incubation medium. These increased levels varied between experiments but were always in a micromolar range above buffer controls. The Mg^{2+} efflux appeared steepest between 5 and 15 minutes, with a lesser rate for up to an hour (data not shown). No reproducible difference could be detected in the Mg^{2+} efflux in the presence of either F-Met-Leu-Phe (Table 5) or EDTA (data not shown). Lactic dehydrogenase (LDH) showed less than 3% enzyme release in all experiments. The latter indicates that the increase in Mg^{2+} was due to the loss of Mg^{2+} from viable cells.

Discussion

We have analyzed the extracellular cation requirements for orientation and movement of rabbit PMNs on glass coverslips in a gradient of the chemotactic peptide F-Met-Leu-Phe. The study was made possible by the development by Zigmond[11] of a chamber that allows the establishment and maintenance of a gradient of the peptide over periods of 1 hour or longer. The coupling of the chemotaxis chamber to a video system permitting rapid analysis not only of orientation but also of cell path length, di-

rection, and speed, as described here, adds a new dimension to the study of chemotaxis by single cells.

In her original description of the chamber, Zigmond reported a time course and dependence on peptide concentration for orientation of PMNs that are compatible with our data for orientation of cells in complete Hanks' medium. Furthermore, Zigmond reported that EDTA and EGTA have no effect on cell orientation toward an optimal concentration of F-Met-Leu-Phe (10^{-8} M peptide). We have confirmed this result with the same optimal concentration of F-Met-Leu-Phe and have extended the analysis to cover a complete range of times and peptide concentrations.

However, our results, with the use of the time-lapse video system, show that the minimal ionic conditions for orientation are not sufficient for cell locomotion. Thus, over a wide range of F-Met-Leu-Phe concentrations and incubation times, no Mg^{2+} or Ca^{2+} requirement could be demonstrated for orientation. On the other hand, cell locomotion was abolished by EDTA, but not Mg^{2+} EGTA, and so appeared to be absolutely dependent on the presence of extracellular Mg^{2+}. The minimal concentration of free Mg^{2+} below which cells showed pulsatile activity at one point without uropod detachment but with lamellipodial extension is extremely low, being around 3×10^{-9} M Mg^{2+}. Consistent with this absolute requirement for a low level of extracellular Mg^{2+}, Bryant et al [18] showed that extracellular Mg^{2+}, but not Ca^{2+}, was necessary for neutrophils to migrate in glass capillaries. They related this to their finding that Mg^{2+} was required for the neutrophils to adhere to glass. Defective adhesion of the whole cell, however, cannot explain our results, since we found no difference in the number of PMNs that adhered to glass and remained attached during routine washing procedures when cells were plated in medium containing or depleted of either Ca^{2+} or Mg^{2+}. However, a selective inability of an advancing lamellipodium to adhere to the substratum in the absence of Mg^{2+} is one possible explanation for our data. An alternative explanation is that Mg^{2+}, probably via its interaction with the cytoskeleton or membranes, is required to enable detachment of the anchoring uropod.

In contrast with Mg^{2+}, exogenous Ca^{2+} was found necessary neither for orientation nor for movement. Indeed, cells incubated with Mg^{2+} in the presence of Ca^{2+} moved more slowly up a gradient of F-Met-Leu-Phe than cells incubated with Mg^{2+} alone. These results are inconsistent with previous evidence from Boyden chemotactic chambers that exogenous Ca^{2+} is required for PMN locomotion.[19,20] One obvious difference between the two systems is that locomotion in the Boyden chamber occurs into or through a filter, whereas PMNs move on a glass surface in the Zigmond chamber. In the former system, demonstration of locomotion may depend

largely on the ability of PMNs to deform during their passage through narrow pores. In contrast, the rate of locomotion on glass may depend on quite different factors, for example, the balance between PMN adhesiveness to glass that might retard movement versus their motile response to the chemotactic gradient. Indeed, a synergistic effect on rat neutrophil adhesiveness to serum coated glass beads has been shown to occur in the presence of both extracellular Ca^{2+} and Mg^{2+}.[21] Whether such differences can account for the discrepant results with respect to exogenous Ca^{2+} requirement remains for future work.

We emphasize that the independence of cell locomotion on exogenous Ca^{2+} does not, of course, rule out the possibility that specific changes in levels of free endogenous Ca^{2+} are essential for orientation, cell movement, and/or the recruitment of contractile microfilaments to the leading edge of motile PMNs. In particular, Naccache et al[22] have shown a time-dependent decrease in fluorescence due to chlorotetracycline when neutrophils are exposed to chemotactic factors. This decrease of fluorescence could be attributed to the loss of Ca^{2+} from cell membranes, including, but not restricted to, the plasma membrane. The fluorescence decrease occurred whether or not the incubation medium contained Ca^{2+}.

In summary, we have demonstrated a clear difference between the divalent cation requirements for orientation and movement of rabbit PMNs across glass coverslips up a gradient of F-Met-Leu-Phe. This distinction was made possible by time-lapse video analysis of cells moving in the chemotaxis chamber developed by Zigmond. The addition of this or other video systems to the laboratory microscope can be simply achieved. The present expansion of computer technology to video systems provides immediate routes for the improvement of the speed and sophistication of future analyses.

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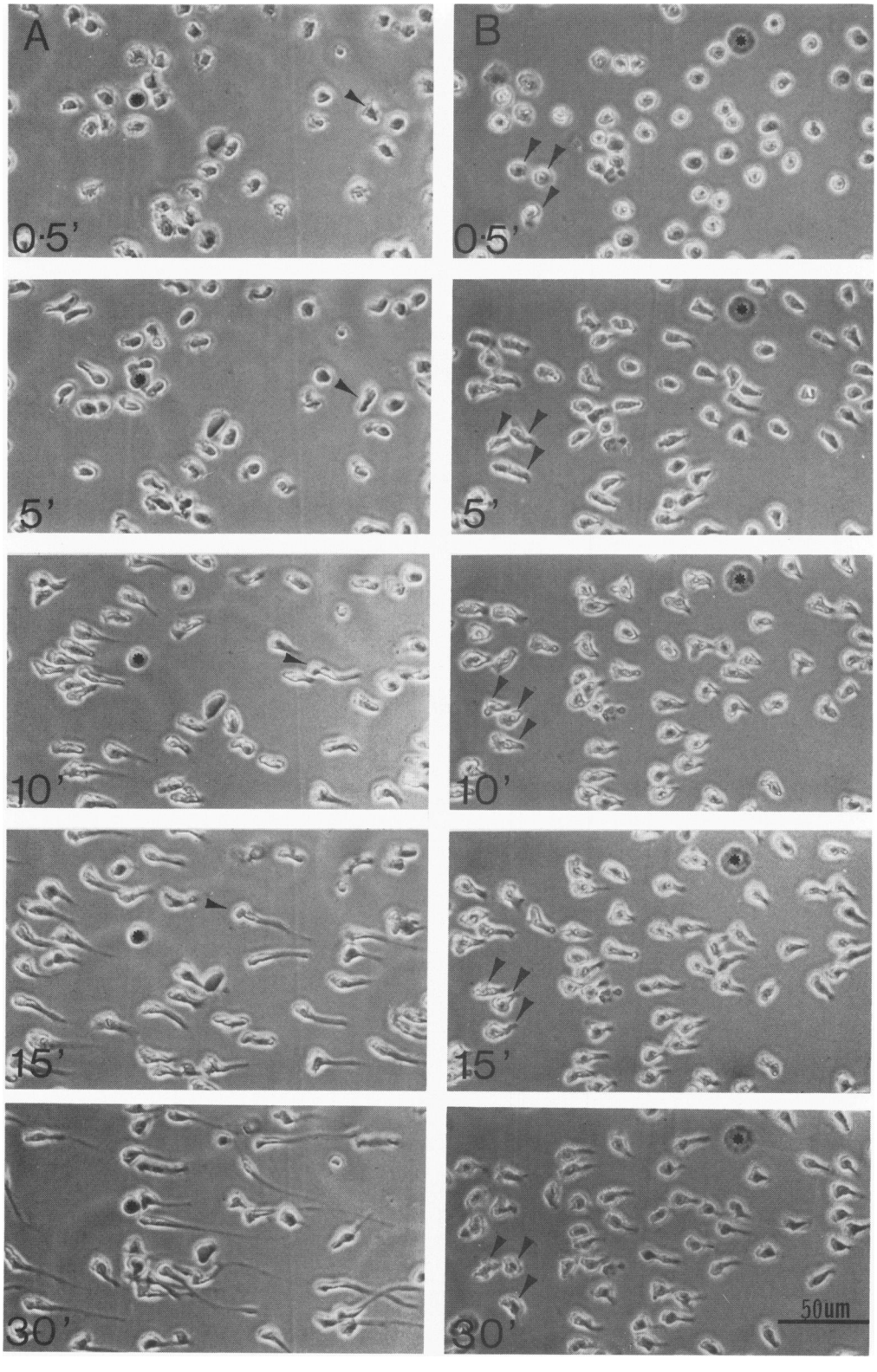
[*Illustrations follow*]

Figure 1—The effects of cations on PMN locomotion. Panel A shows 5 successive photographs of cells exposed to a gradient of 10^{-8} M F-Met-Leu-Phe in complete medium. An immobile reference cell is marked with an *asterisk*. Cell shape changes are evident by 5 minutes after establishing the gradient, and extensive orientation is established by 10 minutes. Orientation is maintained at 15 and 30 minutes of incubation. Cell movement follows orientation. Thus the cells marked with arrows change shape but not position on the coverslip over 5 minutes of incubation of F-Met-Leu-Phe. At longer times, movement is clearly seen. Panel B shows successive frames from chambers run identically, except that EDTA is present to chelate Mg^{2+}. The rate and extent of cell orientation is identical to that seen in complete medium. However, uropod length is somewhat reduced, and it is clear that no locomotion is occurring. For convenience, three cells that show repetitive shape changes without locomotion (see text) over 30 minutes are marked with *arrows*. The *asterisk* marks an immobile reference macrophage. The figure is a composite of working prints used to obtain the data shown in Text-figure 4. Photography on Kodak Tri-X-Pan film. (×200) (With a photographic reduction of 20%)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