ABILITY OF POLYMORPHONUCLEAR LEUKOCYTES TO ORIENT IN GRADIENTS OF CHEMOTACTIC FACTORS

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ABSTRACT

Polymorphonuclear leukocyte (PMN) chemotaxis has been examined under conditions which allow phase microscope observations of cells responding to controlled gradients of chemotactic factors. With this visual assay, PMNs can be seen to orient rapidly and reversibly to gradients of *N*-formylmethionyl peptides. The level of orientation depends upon the mean concentration of peptide present as well as the concentration gradient. The response allows an estimation of the binding constant of the peptide to the cell. In optimal gradients, PMNs can detect a 1% difference in the concentration of peptide.

At high cell densities, PMNs incubated with active peptides orient their locomotion away from the center of the cell population. This orientation appears to be due to inactivation of the peptides by the cells. Such inactivation in vivo could help to limit an inflammatory response.

KEY WORDS chemotaxis · leukocytes · gradients · polarity · *N*-formylmethionyl peptides

Polymorphonuclear leukocytes (PMNs) are among the most motile mammalian cells and are capable of locomoting at rates >30 μm/min. In addition, PMNs can orient their locomotion in a chemical gradient, i.e., exhibit chemotaxis. Their ability undoubtedly facilitates the accumulation of PMNs at sites of injury or infection. Examination of the conditions under which a leukocyte can detect the direction of a chemical gradient may outline general requirements for determining polarities of many cells during embryonic morphogenesis and adult tissue turnover.

Most current studies of leukocyte chemotaxis utilize variations of a millipore filter assay system introduced by Boyden (8). In these assays, PMNs are allowed to migrate into the pores, usually 3 μm in diameter, of the millipore filter which is placed between two chambers. By varying the solution placed in the two chambers, a gradient is established across the filter. Studies using these systems have identified a number of chemotactic agents for PMNs, including serum components, cell-derived materials, denatured proteins, bacterial factors, and certain *N*-formylmethionyl peptides (32, 29). The millipore assays have demonstrated that there is a dose-dependent stimulation of locomotion (chemokinesis) as well as directed locomotion (chemotaxis) by these chemotactic factors.

Although permitting impressive advances, the millipore technique has certain limitations. The evaluation of chemotaxis is indirect and must be calculated after correcting for variations in the level of random locomotion. Due in part to this, the assay appears to be most sensitive with rapidly moving cell populations. Incubation times are generally long (between 30 min and 3 h), making the evaluation of initial or transitory effects difficult. Most importantly, it is not possible to observe the cells as they are responding. Observational studies can determine whether a given alteration in the

chemotactic response is due to variations in (*a*) the percentage of cells responding, (*b*) the accuracy of the orientation, (*c*) the frequency or magnitude of turns, or (*d*) selective changes in the rate of movement by cells in a certain orientation relative to the direction of the gradient.

Leukocyte chemotactic behavior in known concentrations and concentration gradients of chemoattractant has never been adequately studied. Several attempts to study the cell responses have been fraught with problems such as the failure to get a positive response to any soluble factor tested (19), insufficient information on the nature of the gradients established (28), or results indicating no variation in chemotactic responsiveness over 10,000-fold range in concentration of chemotactic stimulant (18).

Using a new system in which cells can be viewed by phase microscopy as they are exposed to controlled concentrations and concentration gradients of chemotactic factors, I have investigated the ability of PMNs to orient along the direction of a gradient of two *N*-formylmethionyl peptides. In this paper, I show that cell orientation depends upon the mean concentration as well as the concentration gradient of the factors, thus deviating from the classical Weber-Fechner law of sensory physiology. In certain concentration ranges, the cells can detect a concentration difference across their dimensions of ~1%. Cells appear to inactivate the chemotactic factors and can be seen to respond to the local gradients thus created. The orientation can occur in the presence of antimitotic agents such as colchicine, and in the absence of external protein or divalent cations.

MATERIALS AND METHODS

The Chamber

The chamber design was suggested by the conditions developed for studies of transport across membranes; when a permeable or semipermeable membrane separates two stirred solutions of infinite volume, a stable linear gradient is established across the membrane (14). To mimic these conditions as closely as possible while being able to visualize cells exposed to the gradient, a 1″ × 3″ × 1/8″ Plexiglass slide was cut to have two wells 1 mm deep and 4 mm wide separated by a 1-mm bridge (Fig. 1). A 22 × 40 mm cover slip over the bridge and wells was held firmly in place with a brass clip screwed into the Plexiglass at each end. The chamber was assembled by inverting a cover slip with cells attached across its center region onto the Plexiglass slide and securing the cover glass in place with the clips. The two wells (each holding ~100 μl) were then filled with different concentrations of chemotactic peptide in a buffer solution.

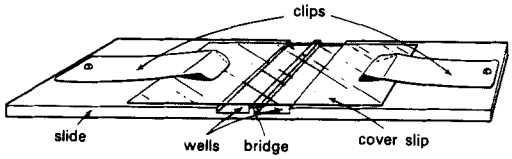

FIGURE 1 Chamber used for visual assay.

If both ends of the cover slip are clean and dry when it is placed on the slide, the layer of fluid over the bridge is very thin (only those preparations were scored in which the distance between the top of the bridge and the bottom of the cover slip, measured with the micrometer on the fine focus knob, was between 3 and 10 μm thick). The resistance to flow over the bridge is sufficiently great that one well can be filled with a solution containing trypan blue without detectable amounts of the dye flowing into the other well filled with water. The trypan blue well remains distinctly darker than the water well, even after 72 h at room temperature.

From diffusion equations, one can calculate that in the absence of flow, a linear gradient of a molecule of ~450 mol wt will be established to 99% completion across the bridge in 76 min (20). Because some convection occurs as the wells are filled, the development of the gradient was evaluated experimentally with a fluorescent probe, fluorescein isothiocyanate.

Fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, Mo.) was dissolved in water and adjusted to pH 10 with sodium hydroxide to optimize the yield of fluorescence. After the wells were filled with varying concentrations of FITC, the bridge was photographed through a ×16 objective on a Zeiss epifluorescence scope with a mercury lamp. The Tri-X film was developed with 1:1 dilution of Kodak D-76. The density of the negative was scanned on a Joyce-Loebl densitometer (Joyce, Loebl and Co., Ltd., England) with a 3.0 OD optical wedge. The density of the film after 1-min exposures was proportional to the log of the concentration of FITC between 2×10^{-4} and 10^{-5} M on the bridge. When the wells are filled with different concentrations of FITC, a gradient can be detected 1 min after the wells are filled (Fig. 2). The gradient is steepest between 15 and 90 min. After 90 min at room temperature, there is often a decline in the steepness of the gradient. Variations among experiments indicated that it was not possible to predict the exact shape of the gradient at any given time; nevertheless, between 30 and 90 min the gradients were usually steep and stable.

Chemotactic Factors

The chemotactic factors used were *N*-formylmethionylmethionylmethione (FMMM) and *N*-formylmethionylleucylphenylalanine (FMLP) which were the generous gifts of Dr. E. Schiffman of the National Institutes of

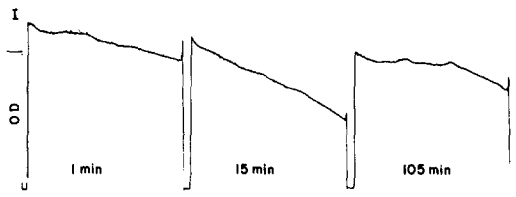

FIGURE 2 Densitometer tracings of films of the bridge of the chamber at various times after one well was filled with 1×10^{-4} M FITC and the other well with 1×10^{-5} M FITC.

Health, and Dr. E. L. Becker of the University of Connecticut Medical Center at Farmington, respectively. The factors were stored at −20°C as concentrated stock solutions in Hanks' basic salt solution or in dimethyl sulfoxide. Unless otherwise specified, assays were done in Hanks' balanced salt solution (HBSS) containing 1% Knox gelatin (Kind & Knox Gelatin Co., Cherry Hill, N.J. [Hanks-gel]). Dimethyl sulfoxide controls at the maximum concentrations used (0.1%) were negative.

Chemicals

Colchicine, soybean, and lima bean trypsin inhibitors, L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), *N-p*-tosyl-L-lysine chloromethyl ketone HCl (TLCK) and *N*-benzoly-L-tyrosine ethylester (BTEE) were obtained from Sigma Chemical Co. Human alpha 1 antitrypsin was obtained from Worthington Biochemical Corp., Freehold, N. J.

Cells

HUMAN CELLS

CLOT PREPARATIONS: Drops of fresh blood obtained from a finger prick were placed on cover slips and allowed to clot. After a 1-h incubation at 37°C in a moist chamber with 5% CO_2, the clot retracts and the cover slips can be gently washed with saline to remove the clot and red blood cells. This procedure leaves granulocytes and monocytes attached to the cover slip.

CELL SUSPENSIONS: Human peripheral blood obtained by venipuncture was placed in an equal volume of 2% Dextran (high fraction, J. T. Baker Chemical Co., Phillipsburg, N.J.) in saline (0.9% NaCl) containing 20 U/ml sodium heparin (grade 1, Sigma Chemical Co.). The red blood cells were allowed to sediment for 15 min before the white cell-rich supernate was removed. The white cells were sedimented at 800 *g* for 5 min. The cells were suspended in saline, and the remaining red blood cells were lysed by reducing the osmotic strength fourfold for 45 s by addition of water. The osmotic strength was readjusted with ×10 saline before the cells were centrifuged again. After centrifugation, the cells were suspended in Hanks' medium and allowed to attach to the center of a 22 × 40-mm cover slip for 10 to 15 min before the cover slip was inverted over the Plexiglass chamber such that the cells lay over the bridge. The brass clips were then placed on the cover slip, holding it firmly in place.

Rabbit PMNs were obtained from peritoneal exudates induced for 4 h by injection of 250 ml of 0.01% shellfish glycogen in saline as previously described (37).

Scoring of Cell Orientation

The cells in the center microscope field of the bridge were observed with a ×40 phase objective (field diameter 0.4 mm). The direction of locomotion of the PMNs was morphologically judged; the front of a locomoting cell was identified by its pseudopod and the rear by its knob-like tail. Cells were scored as either moving into the 180° sector toward the high concentration of a chemotactic factor, or into the 180° sector toward the low concentration. Only cells with pseudopods and tails and whose direction of movement could be evaluated were scored. Thus, immobile cells and cells moving perpendicular (~10°) to the gradient were not counted. The unscored population was usually <20% of the PMNs present. The bridge was scanned across the slide until at least 100 cells were scored. The results were expressed as the number of cells moving toward the high concentration divided by the total number of scorable cells–(the sum of those scored as moving toward and away from the chemotactic factor) × 100. In some cases, the cells were photographed, and the exact angle of the cell orientation was measured in the photograph.

Millipore Assays

The millipore filter assay system was used as previously described (39). Cells suspended at 5×10^6 cells/ml in varying concentrations of peptide in Hanks' medium containing 0.1% bovine plasma albumin (Metrix division of Armour Pharmaceutical Co. Chicago, Ill.) were placed above a 3-μm pore-size Millipore filter (Millipore Corp., Bedford, Mass.) in a Sykes-Moore chamber (Bellco Glass, Inc., Vineland, N. J.). A similar cell-free solution containing the same or a different concentration of peptide was placed below the filter. The chambers were incubated for 90 min at 37°C in 5% CO_2. The filters were then fixed, stained, and processed as described (39). By using the micrometer on the fine-focus knob of the microscope, the distance from the top of the filter to the farthest plane of focus still containing two cells was measured. The distance that cells moved in various uniform concentrations of peptide was used to calculate how far a hypothetical population of cells randomly locomoting would be expected to move in any given gradient (39).

Colchicine Studies

Human or rabbit cells in suspension were incubated at 37°C for 45 min in Hanks' medium containing 100 μg/ml colchicine. The cells were then centrifuged and allowed to settle on a cover slip for 10 min, again in

Hanks' medium containing 100 μg/ml colchicine. The cover slip was then inverted over the bridge of the chamber. The wells of the chamber were filled with Hanks'-gel and 100 μg/ml colchicine with or without 10^{-8} M FMLP. Cell orientation was scored between 20 and 40 min.

RESULTS

Cell Orientation

The cells showed marked orientation toward a well containing 10^{-5} M FMMM when the other well contained buffer (Fig. 3). When the gradient across the bridge was between 10^{-6} and 10^{-5} M FMMM, more than 90% of the PMNs present were locomoting, and >90% of these oriented into the 180° sector of the well containing the higher concentration of FMMM.

Time Course of Cell Response

Cell orientation was scored between 0 and 120 min after filling the wells with Hanks-gel and 10^{-8} M FMLP in Hanks-gel (Fig. 4). The orientation toward the FMLP was apparent within 15 min and maximal between 20 and 30 min. It then remained nearly constant until between 90 and 120 min when some deterioration of the orientation was seen (not shown). The time course of the cell response in general reflected that of the establishment and decline of the gradient as described in Methods.

Concentration Dependence of Cell Orientation

Previous studies have shown that PMNs sense the direction of a chemical gradient by means of a spatial mechanism, detecting a concentration difference across their dimensions (27, 37). If a cell detects a difference in the amount of peptide bound to its surface on different sides, one would expect that the cell could best detect a gradient near the peptide concentration which corresponds to the binding constant of the peptide to the cell surface. It is in the concentration range of the binding constant, K_d, that a given (logarithmic) increase in the concentration of peptide would result in the maximal increase in the percentage of receptors filled. To determine whether or not the orientation by PMNs to a standard gradient depends upon the mean concentration of peptide present, 3- and 10-fold gradients of FMMM were established across the bridge in the concentration range between 10^{-8} and 10^{-4} M. As seen in Fig. 5,

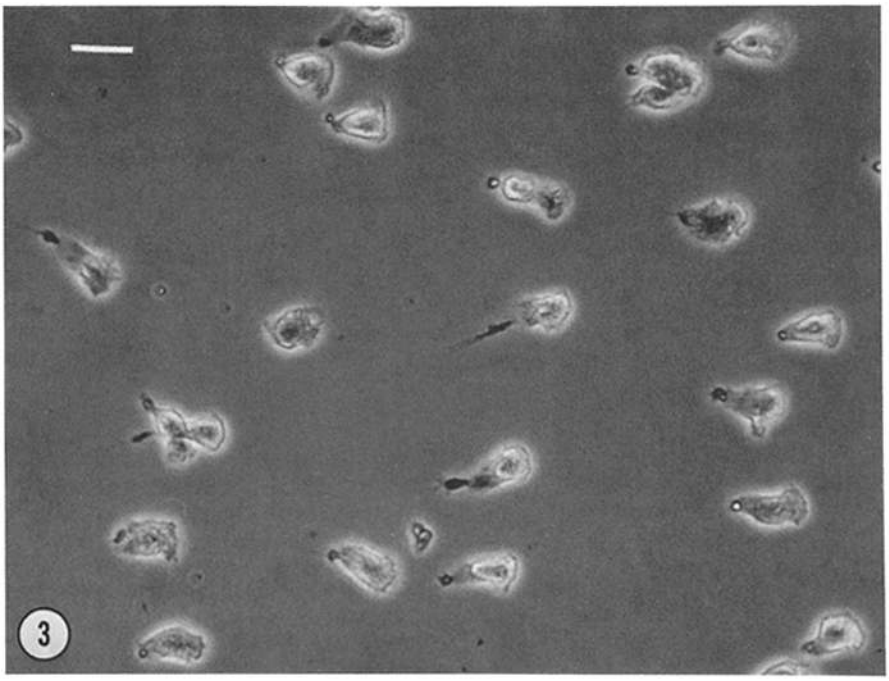

FIGURE 3 Phase micrograph of oriented human PMNs after 30 min in a gradient from 0 on the left to 10^{-5} M FMMM on the right. Bar, 15 μm.

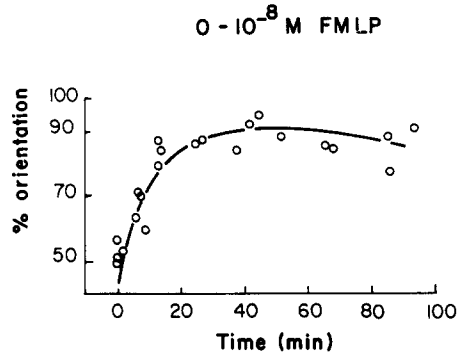

FIGURE 4 Time-course of the development of cell orientation in a gradient from 0 to 10^{-8} M FMLP across the bridge. The percentage of cells oriented toward the well containing FMLP was scored at varying times after the wells were filled, as described in Materials and Methods.

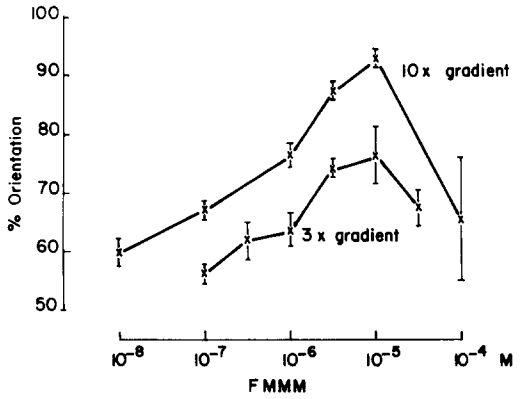

FIGURE 5 Orientation as a function of concentration of FMMM. The orientation of cells exposed to a 10-fold (upper curve) or threefold (lower curve) concentration gradient across the bridge was scored. The concentrations on the abscissa indicate the high concentration of FMMM present in the gradient being scored. The results are presented as the mean percentage of cells oriented plus or minus the SEM (n = 5, 11, 10, 4, 8, and 2 for the tests of increasing concentrations in upper curve, and n = 4, 3, 4, 3, 4, 3 for the tests of the lower curve).

cell orientation to either a 3- or 10-fold gradient was optimal between 10^{-6} and 10^{-5} M FMMM. Thus, the PMN response is a function of the mean concentration of peptide present.

The optimal concentration range remained between 10^{-6} and 10^{-5} M FMMM when the cell concentration over the bridge was between 50 and 1000 cells/mm^2, and for assay times between 30 and 120 min.

A similar concentration dependence of leukocyte chemotaxis has been observed using a millipore assay. As seen in Fig. 6, there was a marked stimulation of rabbit peritoneal PMN locomotion between FMMM concentrations of 10^{-7} and 10^{-6} M. Fig. 6*a* shows that chemotaxis to a 10-fold gradient across the filter increases as the concentration of FMMM below the filter increases to 10^{-6} M. Further increases in concentration resulted in a decreased level of chemotaxis (not shown). Human peripheral blood PMNs showed a dose response in the millipore system similar to that of rabbit cells with an optimal response between 10^{-7} and 10^{-6} M FMMM, even though the same preparation of cells tested in the visual assay showed optimal orientation between 10^{-6} and 10^{-5} M FMMM.

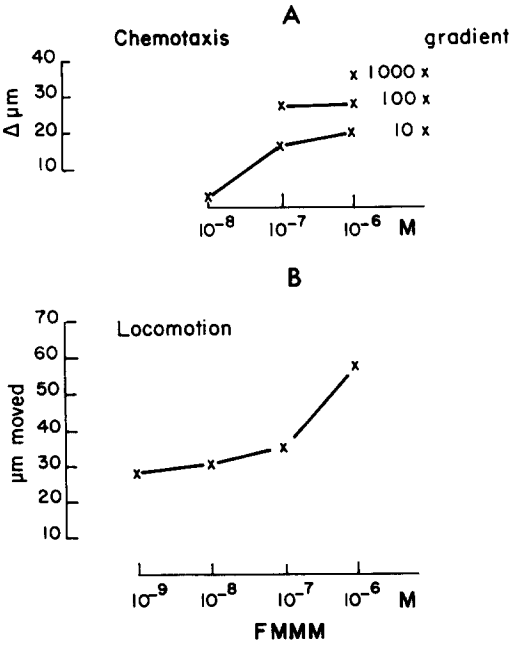

FIGURE 6 Stimulation of locomotion and chemotaxis by FMMM as seen in a millipore assay. (A) Concentration dependence of chemotaxis by rabbit peritoneal PMNs. Chemotaxis is indicated by the increase in distance moved by the front of the cell population with a 10-, 100-, or 1000-fold gradient across the filter in 90 min over the distance a hypothetical randomly locomotion population of cells would be expected to move given the rates of locomotion in varying concentrations of FMMM as shown in Fig. 6 B. The concentrations of FMMM placed below the filter are indicated. (B) The distance to the front of the cell population during a 90-min assay with the same concentration of FMMM present above and below the filter. These assays were run simultaneously with the gradient assays presented in Fig. 6 A.

Sensitivity of the Response

I next examined the minimal concentration gradient required for cell orientation. It follows from the concentration dependence previously described that the ability to sense a shallow gradient will be optimal in the region of the binding constant, K_d, of the factor to the cell. Cell orientation was scored in decreasing gradients in the concentration range between 10^{-5} and 10^{-6} M FMMM. The cell response was usually >90% with a 10-fold gradient across the bridge, and remained significant with a twofold gradient at 10^{-5} M FMMM (Fig. 7). A twofold gradient is a 100% increase in concentration across the 1-mm bridge and a 1% increase over the 10-μm diameter of a cell. Thus, PMNs can orient in a gradient where the difference in concentration on their two sides is 1% and orient efficiently when the difference is 10%. The ability of a PMN to detect a 1% difference in concentration is consistent with estimates from other studies (18, 28).

As the percentage of correctly oriented cells increased, so did their accuracy. Photographs of cells in various gradients were analyzed to determine the angle of orientation relative to the direct line (perpendicular) to the well containing the higher concentration peptide. As the percentage of cells oriented into the correct 180° sector increased, the average orientation of the cells clustered more closely around an angle of 0° (Fig. 8). The increased accuracy is consistent with orientation being initiated by a localized membrane response of the most highly stimulated region of the cell.

Chemotaxis assayed in the millipore system has also been shown to depend on the steepness of the gradient. A 1000-fold gradient of peptide across the filter gave higher levels of chemotaxis than a 100-fold gradient which, in turn, was better than a 10-fold gradient (Fig. 6*a*). Without requiring multiple tests to obtain a significant result, the minimal gradient in which oriented locomotion could be detected in the millipore assay, was a threefold gradient across the filter. Because the filter is ~100 μm thick, this is a 30% difference in concentration across the given cell. Thus, the visual assay appears to be a more sensitive test of the minimal gradient required for orientation than the millipore assay.

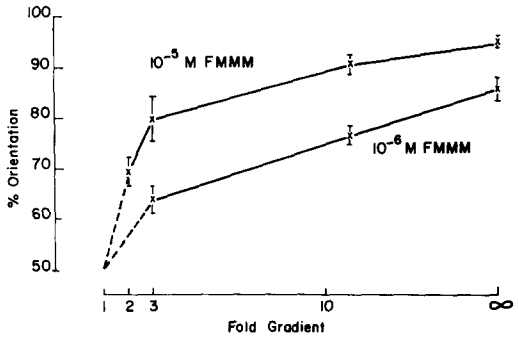

FIGURE 7 Sensitivity of the chemotactic response. 2-, 3-, 10-fold and infinite (buffer in one well) gradients of FMMM were established across the bridge with the high concentration of FMMM being either 10^{-5} or 10^{-6} M FMMM. (n = 4, 2, 8, and 7 for 10^{-5} M FMMM, and n = 3, 10, and 9 for 10^{-6} M FMMM).

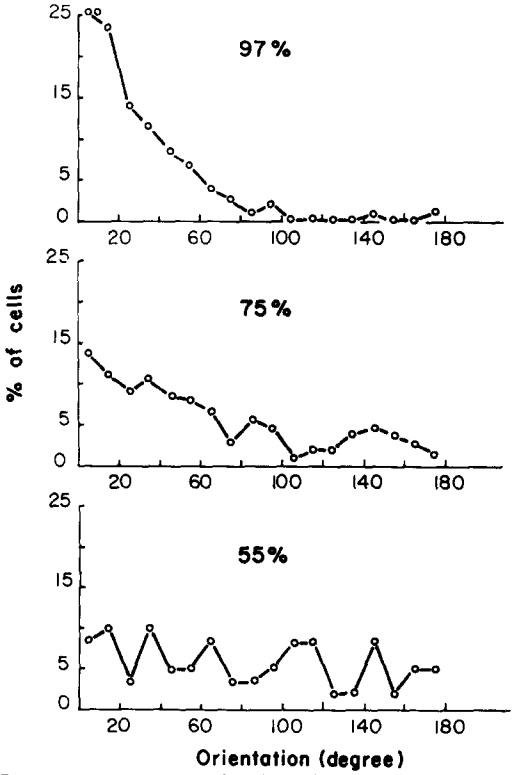

FIGURE 8 Accuracy of orientation. Photographs of preparations with high (97%), medium (75%), or low (55%) percentages of cells oriented in gradient of FMMM were examined to determine the angle of deviation of each cell's orientation from the direct path to the well containing the higher concentration FMMM. Over 100 cells were examined for each level of orientation. The percentage of cells oriented in each 10° sector is plotted.

Reversibility

The orientation response to FMMM was readily reversible. Cells were allowed to orient in a gra-

dient and the orientation was scored. The fluid from both wells was then removed and replaced with fresh solutions such that the direction of the gradient was reversed. The cell orientation was then scored again. Fig. 9 shows that cells were initially oriented with a mean of 86% going toward 10^{-5} M FMMM in the left well (10^{-6} M FMMM was in the right well). 15 min after the gradient was reversed, a majority of the cells (76%) was again oriented toward the 10^{-5} M FMMM now in the right well.

Competition between Factors

The similarity in the structures of various chemotactic *N*-formylmethionyl peptides suggests they might compete with one another as chemotactic stimuli. To test for competition, the ability of increasing concentrations of one factor, which is homogeneously present throughout the system to inhibit the chemotactic response to a gradient of a second factor, was examined. I used a gradient of the second factor steep enough to give a good orientation response, but not so steep that the cell response would be insensitive to decreases in the gradient.

PMNs exhibit optimal orientation toward FMLP between 10^{-9} and 10^{-8} M. As seen in Fig. 10, the cell orientation to a 10-fold gradient (10^{-6} to 10^{-5} M) of FMMM decreased from 82 to 69% by having a homogeneous concentration of the 3 × 10^{-9} M FMLP present.

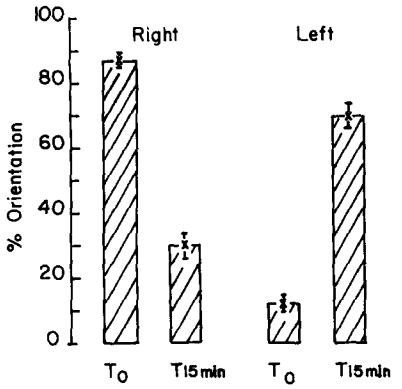

FIGURE 9 Reversibility. Cells were scored for orientation after a 30-min incubation (labeled T_0) in a gradient from 3 × 10^{-7} to 3 × 10^{-6} M FMMM in the right well. The fluid in the wells was then removed and replaced with fresh medium with the direction of the gradient reversed. Orientation toward the 3 × 10^{-6} M FMMM, now in the left well, was scored 15 min after the reversal (T = 15) n = 4 for both groups.

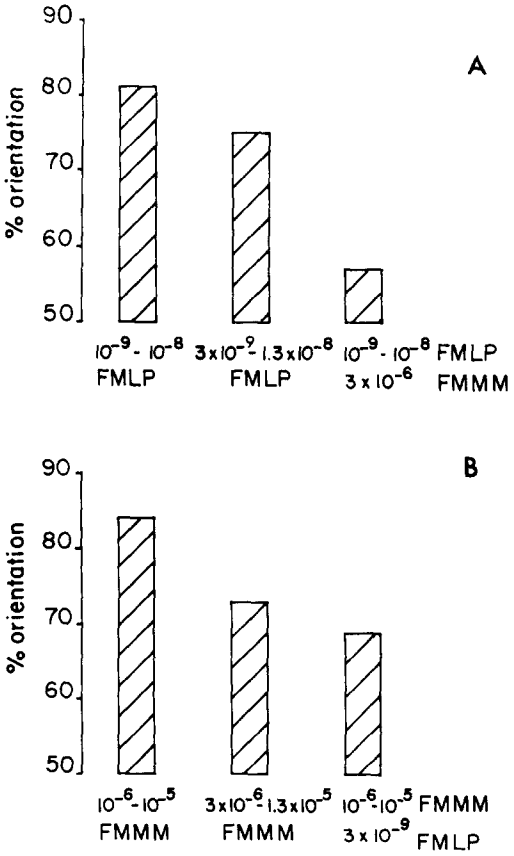

FIGURE 10 (A) Competition. Cell orientation in gradients of 10^{-9} to 10^{-8} M FMLP is shown with no addition, or with the addition to both wells of 3 × 10^{-9} M FMLP (a test of the sensitivity of the assay) or 3 × 10^{-6} M FMMM (a test of competition by a different peptide) to both wells. (B) Reciprocal test of the ability of 3 × 10^{-9} M FMLP homogeneously present in the system to decrease the cell orientation in a gradient of 10^{-6} to 10^{-5} M FMMM.

The reciprocal inhibition could also be observed. The presence of 3 × 10^{-6} M FMMM decreased the cell orientation in a gradient of 10^{-9} to 10^{-8} M FMLP. Presumably, this competition is occurring at a membrane receptor, because recent studies by Aswanikumar et al. (1) and Williams et al. (36) indicate that many of the peptides compete with one another for binding.

N-Formylmethionyl Peptides are Themselves Chemotactic

It is known that under some conditions PMNs release chemotactic factors into the medium. Cells in contact with aggregated gamma globulin or

other denatured proteins release chemotactic factors which then attract other PMNs to the site (39). In addition, high concentrations of chemotactic factors can themselves stimulate release of lysosomal enzymes from PMNs (5, 6, 32).

The possibility exists that the *N*-formylmethionyl peptides are not chemotactic themselves, but rather stimulate, in a dose-dependent manner, cells to release an endogenous chemotactic factor which then induces the cell orientation. This possibility was ruled out by two different experiments. Preparations were set up with the cell population extending only partway across the bridge (no cells were present on the high concentration side of the bridge). The "front cells" could then be scored. These front cells were found to orient to a significantly greater degree ($P = 0.01$) toward a solution of FMMM than toward a buffer solution. Secondly, the dependence of the orientation on cell number was examined. It was reasoned that if a cell-derived material was involved, lowering the cell concentration should lower the amount produced and thus decrease the cell response. Studies examining a chemotactic factor released by cells in contact with aggregated gamma globulin had shown such a cell dependence (38). Various concentrations of cells were allowed to attach to a defined region of the cover slip and then were examined in the chamber. There was no decrease in the orientation as the cell concentration was decreased. In contrast, some decrease in orientation was observed at very high cell concentrations.

Cell Inactivation of Formylmethionyl Peptides

In examining the decreased orientation at high cell concentrations, I noticed that cells on each side of the bridge were oriented toward the well nearest them. This orientation, termed the "edge effect", could be due to inactivation of chemoattractant by the cells on the bridge which would result in a gradient being established from the center of the bridge toward each well which contained a supply of attractant. The edge effect was a function of cell concentration; orientation toward both sides increased when high cell densities were present on the bridge and the wells were both filled with the same concentration of FMMM (Fig. 11). That the edge effect was due to inactivation and not to some physical aspect of the chamber or a negative chemotactic factor released by the cells was indicated by preparations in which buffer was

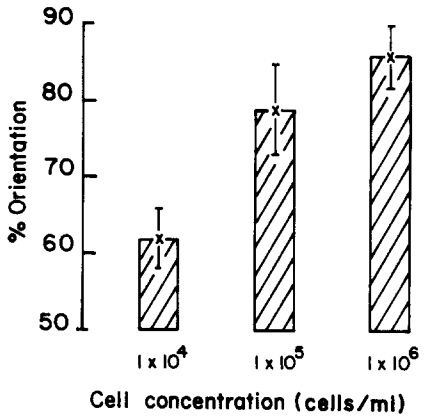

FIGURE 11 The cells within 0.4 mm of either edge of the bridge were scored for orientation toward the adjacent well. Cell concentrations were varied by allowing the cells in 0.1 ml of suspension containing 10^4 ($n = 4$), 10^5 ($n = 4$), or 10^6 ($n = 10$) cells/ml to settle on the cover slip. Cells were obtained from the supernate of dextran sedimented peripheral blood. Both wells contained 10^{-6} M FMMM.

placed in one or both wells. Under these conditions, there was no significant orientation toward the buffer wells even at high cell concentrations.

Aswanikumar et al. (2) have recently obtained chemical evidence that cells can cleave dipeptides between *N*-formylmethionine and the remaining amino acid. The digestion can be inhibited with 0.1 mM TPCK, an irreversible inhibitor, or with 0.1 mM BTEE, a competitive substrate (2, 16). The proteolytic inhibitors tested thus far were not able to inhibit the edge effect without inhibiting cell locomotion. BTEE (1.1 mM) and TPCK (0.1 mM) inhibited locomotion to such an extent that no polarized morphology was observed; at lower concentrations (0.01 mM) of BTEE or TPCK, both chemotaxis and the edge effect were observed. Other inhibitors such as EDTA or ethylene glycol bis (β aminoethyl ether)*N*,*N*,*N*^{1},*N*^{2}, tetraacetate (EGTA) (1 mM) or 1 mg/ml lima bean or soybean trypsin inhibitor, 1 mg/ml human alpha 1 antitrypsin or 0.01 mM TLCK did not inhibit either chemotaxis or the edge effect.

Role of Microtubules

Human PMNs pretreated for 45 min with colchicine (100 μg/ml) and then tested in the presence of colchicine were able to orient in a chemotactic gradient. In two experiments, control cells oriented in a gradient of 0–10^{-8} M FMLP across the bridge at levels of 89 ± 12% (mean SEM, n =

6) and 87 ± 5% (n = 8) while colchicine-treated cells showed 80 ± 13% (n = 6) or 81 ± 10% (n = 11) orientation. Malech et al. (23) have shown that these levels of colchicine effectively depolymerize the microtubules of human PMNs. Conflicting results have been reported on the ability of leukocytes to exhibit chemotaxis in the presence of antimitotic agents (3, 9, 11, 21). The visual observations indicate that leukocytes can orient in a chemical gradient when microtubules are depolymerized but the level of orientation may be decreased.

Medium Requirements

Clot preparations washed with saline containing 1 mM EDTA or EGTA were examined for their ability to orient toward 10^{-8} M FMLP in saline containing either 1 mM EDTA or EGTA. Cells scored at 30 min oriented at normal levels in the presence of either chelating agent. Thus, neither external divalent cations nor protein is essential to the orientation response, although they may be required for cell locomotion and prolonged survival (4, 15, 22, 24, 33, 34, 35).

DISCUSSION

Until recently, pure chemotactic factors were difficult to obtain. In 1975, Schiffmann et al. (29) demonstrated that certain *N*-formylmethionyl peptides were active attractants for PMNs in concentrations as low as 10^{-9} M. These factors can be synthesized in large quantities in pure form, and thus are useful for studies on the chemotactic response. That similar peptides may be physiologically important is suggested by the work of Goetzl and Austen (17), in which extracts isolated from sensitized lung have been shown to contain two tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu, which are chemotactic for eosinophils.

The new visual assay presented here allows rapid evaluation of cell orientation in specified concentrations and concentration gradients of these chemotactic factors. It complements the widely used millipore techniques by allowing analysis of individual cell behavior and morphology during initiation, execution, and reversal of the chemotactic response.

Concentration Dependence of the Orientation Response

With this assay, leukocyte orientation was shown to depend on both the mean concentration and the concentration gradient of the chemoattractants FMMM and FMLP. There was a particular concentration range over which cell orientation to a standard gradient (e.g., 10-fold) was optimal. The concentration dependence of leukocyte chemotaxis can also be seen with millipore filter assays (30, 32, 39). Thus, the leukocyte response deviates from the Weber-Fechner law of sensory physiology which would predict a response solely dependent on the gradient. These observations are consistent with a model in which the chemotactic peptide binds to specific cell receptors, and the direction of the gradient is detected by a difference in receptor occupancy in different regions of the cell surface (39).

If the chemotactic response depends upon detecting a difference in receptor occupancy, the concentration range for optimal orientation should correspond to the concentration of the binding constant, K_d, of the peptide with the cell receptors. In this range, a small change in the concentration of peptide present results in a maximal change in the percentage of receptors occupied. Decreased orientation at lower and higher concentrations of peptide could be due to a failure to detect differences in the percentage of receptors bound when most of the receptors are either empty or saturated. In studies of bacterial chemotaxis, the concentration resulting in optimal chemotactic response has been shown to correspond closely to that of the binding constant of the chemotactic agent with the cell (31). Williams et al. (36) have studied the binding of tritiated FMLP to human PMNs. Under conditions comparable to those used for the behavioral studies (37°C in balanced salt solution), they find a binding constant of $\sim 1 \times 10^{-8}$ M. This corresponds to the estimated binding constant for FMLP from the visual assay of between 10^{-9} to 10^{-8} M.

Millipore assays tend to demonstrate a maximal chemotactic response at concentrations somewhat lower than does the visual assay. Experiments were carried out to determine if the difference in optimal concentration was due to differences in the length of the assay, the cell concentrations, or the species of PMNs used (previous examinations of peptides in millipore assays used rabbit peritoneal cells, and the visual assays, which required fewer cells, routinely used human cells). The same concentration optimum was obtained with the visual assay whether a cell concentration of 500 or 50 cells per mm^2 was used, and whether the cells were assayed at 30 or 120 min. A given preparation of

human cells showed a lower optimal concentration for chemotaxis when tested in the millipore assay than in the visual assay. Thus, none of these factors alone account for the variation, although it is possible that some combination of these and other factors may contribute to the difference. In the visual assay even at lowered cell concentrations, inactivation of peptide by cells in the small volume over the bridge may occur, thereby decreasing the concentration of peptide actually present. Finally, the discrepancy between the two assays may be due to the different parameters which they measure. The visual system measures cell orientation and requires only enough motility for a cell to develop a polarized morphology; the millipore assay measures oriented movement and requires active cell locomotion.

Cell Inactivation of Peptides

In the absence of a gradient, orientation of cells toward the edge of the bridge nearest them (the edge effect) was dependent on high cell concentrations and the presence of peptide in the wells. These observations suggest that the edge effect is due to inactivation of the peptide by the cells through absorption, ingestion, or digestion. Aswanikumar et al. (2) have presented evidence that PMNs are capable of digesting *N*-formylmethionyl dipeptides. Behavioral evidence for inactivation of chemotactic factors has not been previously reported, although Oldfield (26) and Carrel and Ebeling (10), described cells which avoided regions between two centers of high cell density. They believed this phenomenon to be due to a repulsive interaction between the cell populations. However, a depletion of stimulatory agents in regions near high cell concentrations could also be important. Cell inactivation of chemotactic factors in vivo may play a role in limiting the inflammatory response.

Reversibility

Previous studies by Ramsey (27), Cornley (13), and Bessis (7) have demonstrated the reversibility of the direction of leukocyte locomotion. The current studies confirm and quantify these observations. In addition, observation of the movements of cell reversing their direction of locomotion suggests that there is an inhibition of pseudopod formation from the posterior regions of locomoting cells. This inhibition can also be seen in randomly locomoting cells which rarely make a turn of >90° (25). The inhibition probably results in a tendency for cells to maintain a given direction of locomotion, and could help to stabilize the chemotactic orientation once it is established (12).

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