Videoanalysis of Chemokinesis: Characterization of Speed, Persistence and Orientation in an Agarose Assay*

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ABSTRACT

Time-lapse video recording and off-line computer analysis were used to characterize the chemokinetic behavior of individual human neutrophils migrating in an agarose assay. When neutrophils were stimulated with an isotropic concentration of formyl-methionyl-leucyl-phenylalanine (fMLP), they migrated with a mean speed of 9.6 μm per min and oriented at random. The ratio of net displacement to total distance travelled (persistence of locomotion) was 0.66, indicating that neutrophils maintained some directional persistence even in the absence of a gradient of fMLP. The speed and persistence of locomotion index were correlated because both faster and slower cells had high persistence, while only slower cells had low persistence. The orientation angle was independent of both speed and persistence of locomotion. These are the first reported direct measurements of the chemokinetic locomotion of neutrophils using the agarose assay.

Introduction

Chemokinesis is a locomotor response to an isotropically distributed chemical stimulus in the environment. The resulting locomotion lacks definite orientation and involves modulation of a cell’s speed and turning behavior, such that the cell migrates faster and shows an increased tendency for directional persistence as a function of the ambient concentration of a chemical. This is in contrast to chemotaxis; the dose-dependent, oriented locomotor response to a gradient of a chemical. Chemotaxis in human neutrophils is the subject of intense investigation owing to the importance of this response in inflammation (see references 9 and 16 for review).

The relationship between chemokinesis and chemotaxis is not fully understood. To clarify this relationship, quantitative studies were performed of the neutrophils locomotor response, with respect to speed, turning, and orientation, both in the presence of a gradient of formyl-methionyl-leucyl-phenylalanine (fMLP). This report...
presents direct measurements of the chemokinetic locomotion of neutrophils migrating under agarose.

Materials and Methods

Collection of Blood

Venous blood was collected from normal volunteers in accordance with the guidelines approved by the Medical University of South Carolina Committee for the Protection of Human Subjects. Neutrophils were isolated as previously described. Briefly, venous blood was collected in citrate-phosphate-dextrose (CPD) anticoagulant. Neutrophils were isolated by Dextran sedimentation of the red cells followed by density centrifugation through discontinuous gradients of Percoll. Washed suspensions of nucleated cells consisted of 99 percent neutrophils, of which 98 percent of the neutrophils were viable immediately after isolation, as determined by the use of an acridine orange/propidium iodide vital staining procedure. Washed neutrophils were suspended in Hanks Balanced Salt Solution (HBSS)* at 10⁷ per ml and assayed within four hours.

Assay of Chemokinesis

Chemokinesis was assayed in an under-agarose system as previously described. Briefly, a layer of agarose containing 10 nM formyl-methionyl-leucyl-phenylalanine (fMLP) was cast in a petri dish, wells were cut in the gel and 10⁵ neutrophils were placed in each well. Preliminary dose-response experiments showed that 10 nM fMLP produced the maximal radial migration (1 nM: 1.5 ± 0.1 mm, 10 nM: 1.8 ± 0.2 mm, 100 nM: 1.6 ± 0.1 mm, n = 6).

The dishes were incubated at 37°C for one hour, then sealed with Parafilm and arranged on the 37°C heated stage of an inverted microscope so that the video field was just outside of a randomly selected well, at a random location around the perimeter of the well.

A transient gradient existed between the gel and the cell-containing well for a period during the preincubation. The gradient was estimated by preparing an agarose gel which contained 1 mM phenol red (phenylsulfophthalien, MW = 354.37), cutting six wells in the gel and adding 10 μl of HBSS+ to each well. The photometer of an Olympus BHS/PM-10AD photomicroscope was used to monitor changes in absorbance, at 550 nm, of the contents of the wells. The change in absorbance was plotted and the half-time of the absorbance change was estimated.

Video Analysis of Locomotion

Time-lapse video recordings were made using an analog enhanced videomicroscope system which produced a sharp image of the cells despite the presence of the agarose layer. Plastic sheets were placed over the screen of the video monitor and, during playback of the video recordings, the centers of randomly selected cells were marked at 15 second intervals for a period of five minutes. The cell paths were retraced, at one minute intervals, onto the digitizing tablet of a Zeiss Videoplan Image Analysis Computer.

Three fundamental locomotor parameters were measured: Speed, defined as the total distance traveled per minute; Velocity, the "straight-line" displacement per minute; and Orientation Angle, the angle of the velocity vector relative to a line from the center of the cell-containing well through the center of the video field, defined as 0°. Orient-
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Station angles to the left and right of 0° were defined as negative or positive, respectively.

The ratio of velocity to speed was calculated and called the Persistence of Locomotion (Persistence Index, PI). The Persistence Index is an indicator of turning which varies between 1.0 (indicating straight-line motion) and 0.0 (indicating no net displacement).

The analyses of chemokinesis were based on three sets of experiments from three donors, with 30 to 40 cells tracked for each measurement.

Statistical Analysis

Frequency histograms were plotted and statistical analyses were performed using a Zeiss Videoplan Image Analysis Computer. The goodness-of-fit of the data to uniform distributions was determined by a chi square test of the goodness-of-fit. Pearson's Product-Moment (Pearson's r) was used to test for correlations between parameters. Analysis of Variance of the Regression (F-test) was used to test independence between parameters. The relationship between parameters was illustrated using two-parameter scatter-diagrams. Statistical tests were considered significant if p < 0.05.

Results

Figure 1 shows the change in absorbance in the 10 μl volumes of the wells during incubation at 22°C. The half-time for the change in absorbance was approximately one minute, and approached equilibrium after approximately five minutes. A gradient of the slightly larger fMLP molecule (MW 458) would decay at approximately the same rate; therefore, during the observation period, cells were responding to a true isotropic distribution of fMLP.

Chemokinesis

Individual Parameters: The mean speed of neutrophils during chemokinesis was 9.6 ± 3.9 μm per min. The

![Figure 1. Plot of the change of absorbance in wells which were cut in an agarose gel containing 1 mM phenol red and filled with indicator-free buffer. The half-time for the change was approximately one minute and the system approached equilibrium after approximately five minutes.](image)
mean persistence of neutrophils during chemokinesis was 0.66 ± 0.25. If the cells were turning completely at random, values for PI would be uniformly distributed within the 0.0 to 1.0 range. The plot of the frequency distribution of the persistence index (figure 3) appears skewed toward higher PI values. To test whether or not PI values were uniformly distributed, an equation was derived for a line with a zero slope and a y-intercept, equal to the number of cells which would be present in each group if the sample was uniformly distributed. The deviation of the data from this line was tested using the chi square goodness-of-fit procedure. The frequency distribution for PI differs significantly from a uniform distribution (chi square = 45.8, df = 9, p < 0.01), suggesting that neutrophils tended to maintain their direction of migration (higher PI) even in the absence of a gradient. The mean orientation angle of neutrophils during chemokinesis was 16.48° ± 105.7°. The very large standard deviation indicates a high degree of dispersion. The frequency distribution of the orientation angle (figure

![Histogram of chemokinetic speed of neutrophils migrating under agarose in response to 10 nM FMLP.](image)

![Histogram of persistence of locomotion index. The distribution is skewed toward higher PI values, indicating non-random turning. The data differ significantly from a uniform distribution; f(x) = 0.045 N0. N0 = 115.](image)
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Figure 4. Histogram of orientation angle. The distribution of the values fits a uniform distribution; $f(x) = 0.056 N_0$, $N_0 = 115$.

4) appears almost flat, as though the cells were oriented at random. An equation was derived for a sample of the same size which was uniformly distributed across the $-180^\circ$ to $180^\circ$ range. The deviation of the data from this line was tested using the chi square goodness-of-fit procedure. The data does not deviate significantly from the line expected for a uniform distribution of orientation values (chi squared = 16.47, df = 15, $p > 0.05$) suggesting random orientation.

Correlation Analysis

Speed vs. Persistence Index: PI is mathematically related to speed (PI = velocity/speed); therefore, a correlation would be expected. A weak but significant correlation was found ($r = 0.29$, df = 113, $F = 10.32$, df = 1,113, $p < 0.05$). Correlations between mathematically related parameters must be interpreted cautiously. The significant F-test indicates that PI is dependent upon speed. However, figure 5 shows a lack of data points in the lower right (higher speed, lower PI) region, indicating that both faster and slower cells have higher PI, while lower PI cells tend to be slower.

Speed vs. Orientation Angle: Speed and orientation were not correlated and were independent ($r = 0.015$, df = 113, $F = 0.03$, df = 1,113, $p > 0.05$). In figure 6 it is shown that in the absence of a gradient the cells orient at random, regardless of their speed.

Persistence Index vs. Orientation Angle: PI and orientation angle were not correlated and were independent ($r = 0.15$, df = 113, $F = 2.59$, df = 1,113, $p > 0.05$). In figure 7 are shown an increased density of points in the right half of the plot (PI > 0.5), but there is no clustering of higher PI values toward smaller orientation angles.

Discussion

Chemokinesis in the Agarose System

Measurements of Individual Cells in the Agarose System: Several methods have been described for estimating the locomotor parameters of populations of cells.\textsuperscript{11,13} These involve fitting the spatial distribution of a population of cells to an equation which models the cells motion as a biased random-walk or a flux. These methods are limited by the assumptions that must be made about the behavior of individual cells, such as whether the population of cells is homogeneous.
Direct measurements of the locomotor behavior of individual cells avoid this limitation.

Another limitation is the presence of unresponsive, non-motile cells which may equal 50 to 60 percent of a given sample of neutrophils. The agarose assay not only facilitates direct measurement of the behavior of individual cells, but excludes such unresponsive, non-motile cells. Only those cells which respond to fMLP by migrating out of the wells during the preincubation period and are in the video field during the observation period were tracked.

**Why study chemokinesis?** Although a number of investigators have measured the locomotor parameters of individual
neutrophils undergoing chemokinesis,\textsuperscript{1,6,7,10,14} no comparable measurements have been published using the agarose system. This study was part of an investigation of the relationship between neutrophil chemotaxis and chemokinesis in the agarose system and establishes a baseline for that comparison.

**Lifetime of a Transient fMLP Gradient:** Immediately after being placed in the wells, the cells experience a transient gradient of fMLP as the peptide diffuses out of the gel and into the suspending medium. It was shown that a compound of similar molecular weight equilibrates within approximately five minutes. The fMLP probably equilibrates at a similar rate so that the cells still in the well would be in an isotropic concentration of fMLP after approximately five minutes. There is probably a net flux of cells out of the well during the preincubation period, prior to the observation period. However, after the one hour preincubation, no bias in orientation was detected during the five minute observation period (figure 4).

**Speed:** The mean chemokinetic speed of neutrophils was 9.6 \( \mu \text{m} \) per min. Previous studies of chemokinesis have reported a wide range of speeds in response to fMLP (10 nM fMLP, 30 \( \mu \text{m} \) per min);\textsuperscript{6} (10 nM fMLP, 9.5 \( \mu \text{m} \) per min);\textsuperscript{7} (10 nM fMLP, 23.7 \( \pm \) 1.8 \( \mu \text{m} \) per min);\textsuperscript{8} (10 nM fMLP, 6.36 \( \mu \text{m} \) per 40 sec).\textsuperscript{14} These differences may be due to unreconciled differences in experimental conditions used by the different investigators. The agarose system was used because it is a steady-state assay which is not subject to transient fluctuations of the fMLP gradient,\textsuperscript{15} and it separates motile from the previously mentioned non-motile cells.

**Persistence:** Neutrophils tend to continue moving in a given direction with a mean PI of 0.66. The results are consistent with a recent report that neutrophils show a response called klinokinesis; a change in the number and size of turns which the moving cell makes in response to a stimulus.\textsuperscript{8} This response is dose-dependent and biphasic such that sub or supraoptimal doses of chemottractant (specifically fMLP) stimulate migrating neutrophils to make more/wider turns than does the optimal dose.\textsuperscript{14} The concentration of fMLP was used, which produced a maximal motile response.

The tendency to show some directional persistence in the absence of a gradient may be due to the inherent polarity which migrating neutrophils display.\textsuperscript{17} Neutrophil polarity has been shown to be a transient state with a char-
characteristic decay time of approximately 30 seconds that could be considered as the time between turns.

**Orientation Angle:** The lack of a preferred orientation would be expected if the distribution of chemoattractant was isotropic, as would a bias for an orientation away from the cell-containing well as the cells migrated out of that well. Such a bias would produce a peak in the orientation frequency distribution around 0°. However, the frequency distribution of orientation fits the equation for a uniform distribution. If the neutrophil is considered to have an information processing system where a gradient of chemoattractant is the input and oriented locomotion is the output, then chemokinesis is the response to a zero-input condition.

**Correlations Between Parameters**

**Speed vs. Persistence:** A correlation was found between speed and persistence with slow cells showing both high and low persistence but with faster cells tending to show high persistence. Although the weak correlation may merely reflect the mathematical relationship between speed and PI, Keller et al. reported a negative linear correlation between speed and a speed/velocity ratio (1/PI) which they called "klinolocomotion index". They stimulated neutrophils with fMLP in sealed slide-and-coverslip preparations, and found that the higher the mean speed of a sample of cells, the higher their directional persistence (the lower the klinolocomotion index). They calculated that, in their system, if cells could be stimulated to migrate with mean speeds >25 μm per min, no turns would be made.

The "klinolocomotion index" for the data of the present authors would be 1.515 (1/0.66). The linear correlation which Keller et al. (figure 4) found would predict a mean speed of 12 to 15 μm per min for our cells. The present authors found a correlation between PI and speed in the system, but a mean speed of 9.6 μm per min was measured. The differences between the agarose system and the "slide-and-coverslip" may influence the speed of migrating cells. For example, the slide-and-coverslip preparation may provide two glass surfaces for cellular adherence during migration.

**Speed, Persistence vs. Orientation:** During chemokinesis, cells migrate in a manner which has been described as similar to diffusion, but in the agarose system they did not show a bias for the direction away from their "source"; the cell-containing well. If cells showed different degrees of orientation as a function of their speed or tendency to turn, it is possible that one parameter could mask the other. The regression analysis and two-parameter scatter-diagrams (figures 6 and 7) show that orientation is independent of speed and persistence. This baseline data is necessary for quantitative studies of neutrophil locomotion since any systematic directional bias would confound the interpretation of data from chemotaxis experiments. No such systematic bias was found.

**Conclusions**

Incubation conditions were used similar to those previously used to characterize chemotaxis, and it was found that the speed of cells undergoing chemokinesis was less than for cells undergoing chemotaxis. These cells showed some directional persistence but did not show a preferred orientation. It was also found that persistence and orientation angle are not correlated, but that speed and persistence are weakly correlated because faster cells tend to be more persistent. The chemotactic speed (~20 μm per min) and PI (0.88) in the agarose system are much higher than the values
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reported here for chemokinesis.\textsuperscript{3} The authors propose that the relationship between chemokinesis and chemotaxis could be either (1) a \textit{gradual increase in speed and persistence owing to increased orientation} or (2) a response to the gradient involving speed, persistence and orientation simultaneously, e.g., the cells \textit{switch} to a different kind of response.

**References**