CHEMOTAXIS IN VITRO

QUANTITATION OF HUMAN GRANULOCYTE MOVEMENT USING A STOCHASTIC DIFFERENTIAL EQUATION

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ABSTRACT The quantitation of human granulocyte movement using a stochastic differential equation is described. The method has the potential to distinguish both positive and negative chemotaxis. Analysis and information concerning cell movements can be obtained for any point in time and distance for the duration of the experiment.

INTRODUCTION

Several recent studies have reemphasized the importance of chemotaxis in cell-cell interactions, for example, the interaction of eosinophils with neoplastic cells (1), role of immune complexes as chemotactic agents in immunologic phenomena (2) and leukocyte behavior in the inflammatory response (3–5).

Several techniques are currently available for detecting chemotaxis (6–9) but the most popular is that devised by Boyden (10). However criticisms of one form or another have been leveled against this latter technique (ref. 11 for review).

This communication describes a new approach to quantitating chemotaxis. Analysis of cell movements allow us to compute the probability of cells reaching a given point with respect to distance and time. The method has the potential of detecting negative as well as positive chemotaxis and of measuring the chemotaxis as a function of position and time.

MATERIALS AND METHODS

Human granulocytes were obtained using the method reported by Harris (8). Briefly, a drop of blood from a pricked finger was placed on a standard 1 by 3 in microscope slide and incubated at 37°C in a moist atmosphere for 30 min. The clot was carefully removed and the residual red blood cells gently washed off with Hanks' balanced salt solution. The remaining cells, approximately 95% granulocytes, were covered with a drop of medium containing 50% human serum (heat inactivated) and 50% minimal essential medium (Eagle's salts), HEPES buffered, pH 7.2. A capillary tube of 1 mm external diameter, filled with an agar plug (control) or *Escherichia*

coli with agar plug (chemotaxis) was placed upon the slide and its position adjusted until the end of the tube was adjacent to an area of granulocytes. A glass strip, 1 mm thick, 1 in long and 2–4 mm wide was placed 1.6 cm away from the capillary tube on the glass slide. A coverslip was placed on top and the resulting chamber was sealed with wax (equal parts Histowax and Vaseline) on three sides. Additional medium to fill the chamber was added through the fourth side which was subsequently sealed. This method resulted in a chamber of uniform dimensions for use in each experiment.

MATHEMATICAL THEORY AND ANALYSIS

The x-displacement of cells is modeled by the stationary stochastic differential equation:

$$dx_t = m(x_t)dt + \sigma(x_t)dw_t, \tag{1}$$

where $m(x)$ and $\sigma^2(x)$ are the drift and diffusion coefficients, and w_t is a one-dimensional Brownian motion. Such processes are studied in refs. 12–14. $m(x)$ is a measure of the local average displacement and $\sigma^2(x)$ is a measure of the variance of the local displacement. Let $I = [0, \alpha]$, $\alpha > 0$, be a finite interval in which the cell motion is observed, and let us define the function $V(x)$ to be the probability that a cell starting at a point $x \epsilon [0, \alpha]$ will reach the boundary point 0 before it reaches α. In refs. 13 and 14 it is shown that $V(x)$ is a solution of the differential equation

$$m(x)[dV(x)/dx] + \tfrac{1}{2}\sigma^2(x)[d^2V(x)/dx^2] = 0, \tag{2}$$

with the boundary conditions:

$$V(0) = 1 \quad \text{and} \quad V(\alpha) = 0. \tag{3}$$

These boundary conditions imply that the boundary points 0 and α behave like sinks. If the cell starts at 0 it must remain there forever, i.e., with probability one, and if the cell starts at α, it remains there with probability one and hence $V(x)$, the probability reaching 0 before α, is zero.

The solution of Eq. 2 with the boundary conditions in (3) is given by

$$V(x) = \int_x^\alpha B(y)dy \Big/ \int_0^\alpha B(y)dy, \tag{4}$$

where

$$B(y) = \exp\left\{-\int_0^y \frac{2m(\xi)}{\sigma^2(\xi)}\, d\xi\right\}. \tag{5}$$

If we set $m(x) = 0$ for all $x \epsilon [0, \alpha]$, i.e., there is as much tendency to move to the left as to the right from any starting point $x \epsilon [0, \alpha]$, Eq. 4 reduces to

$$V(x) = (\alpha - x)/\alpha \tag{6}$$

i.e., $V(x)$ is a straight line with value 1 at 0 and value 0 at α, denoted by $\mathcal{L}$ in Fig. 1.

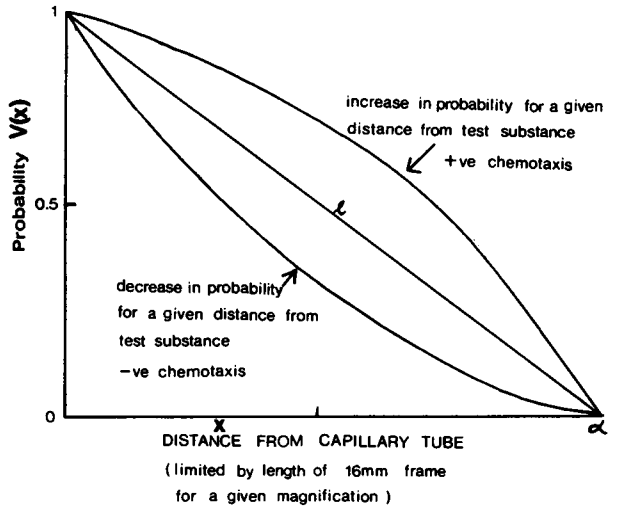

FIGURE 1 Probability $V(x)$ as a function of distance showing neutral and positive and negative chemotaxis.

Under this neutral condition, if a cell starts at the midpoint $x = \alpha/2$, then we would expect that the probability of reaching point 0 before point α is 0.5. This is borne out by Eq. 6.

If an attractant were placed at point 0, then we would expect that on the average the displacements around any point $x\epsilon[0, \alpha]$ would be greater to the left than to the right, i.e., $m(x)$ is negative. For $m(x)$ negative on $[0, \alpha]$, the $V(x)$ curve is above the neutral line ℓ, indicating that the probability of reaching 0 before α is greater than in the case where there is no attractant. A repulsion from 0 would similarly be indicated by $V(x)$ dropping below ℓ.

From these considerations, it can be seen that $V(x)$ serves as a measure of the attractive or repulsive force felt at any point $x\epsilon[0, \alpha]$ by a cell, and can thus be used to compare the attractive or repulsive forces along the slide for different chemotactic attractants or repellents positioned at 0.

It is important to keep in mind that in the above analysis the time-homogeneity of the stochastic differential equation is essential. It may be of interest to know how the attractive or repulsive force varies with time as well as position. To do this, we simply divide up the time interval of observation into smaller intervals in which the assumption of homogeneity is more accurate. In the i th time interval, we define $V_i(x)$ to be measure of attraction or repulsion on the spatial interval $[0, \alpha]$. Comparison of $V_i(x)$ for various i's yields an indication of the time variation of the chemotactic force.

We remark that using the stochastic differential Eq. 1 to describe the cell motion tacitly assumes that the motion is independent of cell number and cell density. Since, as we shall see below, we require only local and very small distance measurements, the

global population may not be an important factor. To treat mathematically the effect of cell population on chemotactic motion is an extremely difficult problem in which the nature of the cell-cell interaction would have to be understood.

To compute $V(x)$, the functions $m(x)$ and $\alpha^2(x)$ are estimated as follows. Fix $\xi \epsilon [0, \alpha]$ and take n measurements, $x_1, \ldots, x_n$ of the horizontal cell displacement from ξ in a fixed short time interval Δt. The random variables $x_1, \ldots, x_n$ form a statistic of size n and allow us to estimate the quantities $E\{\Delta x_{\Delta t}/x_t = \xi\}$ and $E\{(\Delta x_{\Delta t})^2/x_t = \xi\}$, where x_t is the position of the cell at time t and $\Delta x_{\Delta t}$ is the change in horizontal displacement in the time interval $[t, t + \Delta t]$. The standard estimators are $\hat{m}(\xi) = (1/n)\sum_{i=1}^{n}(x_i - \xi)$ and $\hat{\sigma}^2(\xi) = (1/n)\sum_{i=1}^{n}(x_i - \xi)^2$. Hence,

$$\hat{m}(\xi)/\hat{\sigma}^2(\xi) = \sum_{i=1}^{n}(x_i - \xi) \Big/ \sum_{i=1}^{n}(x_i - \xi)^2$$

is an estimator of

$$E\{\Delta x_{\Delta t}/x_t = \xi\}/E\{(\Delta x_{\Delta t})^2/x_t = \xi\}$$

which in turn is approximately equal to $m(\xi)/\sigma^2(\xi)$, the function required in Eq. 5. The quantity $\hat{m}(\xi)/\hat{\sigma}^2(\xi)$ is computed at various points $\xi_1, \ldots, \xi_N$ along $[0, \alpha]$. With this information, functions $B(y)$ and $V(x)$ can be computed numerically.

MEASUREMENT OF CELL MOVEMENT

Cells were filmed using a Wild M40 inverted microscope and a Wild time-lapse unit (Wild du Canada, St. Lambert, Quebec). One frame was exposed every 8 s with an exposure time of 1.5 s using an electromagnetic shutter. The electromagnetic shutter eliminated any possibility of the light affecting cell movements through heating or phototropic effects. Total magnification used was 54X. The preparation was kept at 37°C by a Wild hotstage. Cell densities were selected in the range 90–120 cells per 16 mm frame for a constant magnification. Such a density gave a maximum number of cells for analysis while providing sufficient space for cells to move without hindrance from other cells. Films were analyzed with a Kodak analytical projector (L-M Photo, Van Nuys, Calif.) at a constant magnification.

It was determined that the shortest time interval which could be routinely used in which the cells moved a distance that could be measured with reasonable accuracy was 10 frames (80 s). Consequently, in all experiments, the distance moved in 10 frames was recorded. The approximate center of the cell was taken as the reference point to determine the distance moved. A minimum displacement of one-half a cell diameter was required before a cell was deemed to have moved. The x component of this movement was recorded and for computer analysis $-ve$ x was taken as being toward the capillary tube and positive x away from it (cf. Fig. 2). These x components were measured in millimeters where 1 mm = 4.1 μm actual distance. Cells moving at, or nearly at, right angles to the chemotactic gradient (i.e., parallel to the end of the capillary tube) presented a problem when assigning values to the component of their move-

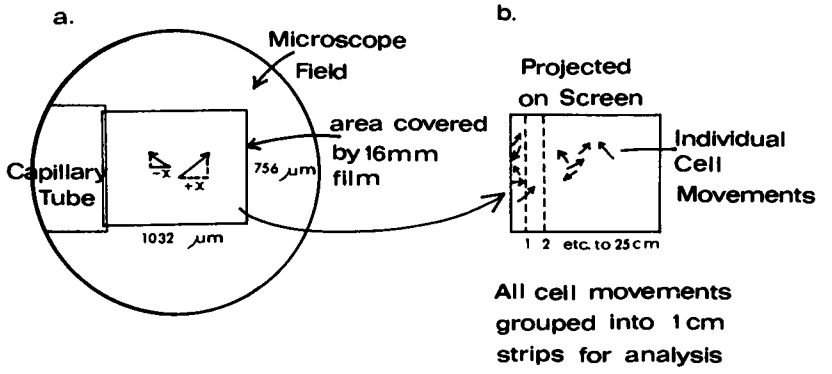

FIGURE 2 (*a*) Area of chamber covered by microscope field and 16 mm film (not to scale). (*b*) Area projected on screen at constant magnification.

ments. In order to overcome a subjective assignment, all x components of cell movements <3mm (<12mm which is approximately half a diameter of a neutrophil attached to glass) were recorded as zero displacements.

The area of the projected film was divided into 25 1-cm strips and all cell movements originating within a given strip were allotted to that strip for analysis (Fig. 2). All subsequent references to distance refer to that strip number.

It will be apparent that the greater the number of cell movements analyzed for a given strip the more accurate will be the statement concerning the probability $V(x)$ for a given distance and time. We wished to determine if we could get sufficient data from one film or whether several separate experiments had to be undertaken and the cell movements for a given distance at a specific time pooled. Consequently, initial analyses of cell movements in the control (neutral) situation were carried out in two different ways. In method A, one film was made and measurements recorded on cell movements on multiple 10 frame units staggered as follows: 0–10, 2–12, 4–14,..., 10–20, giving six 10 frame units within a 20 frame (2 min 40 s) block. This protocol was repeated at the times indicated (see Results) after initiation of the experiment.

In method B, six separate films were produced of six separate control (neutral) experiments and the data from a single 10 frame unit at 80 min after initiation of the experiment were pooled. All chemotaxis data were obtained using method A after the control data using the two methods had been evaluated.

RESULTS

Fig. 3 (*a–e*) shows the probability $V(x)$ as a function of distance copied from the computer printout, for cells moving in the absence of bacteria (neutral case) at the indicated times using method A. Fig. 3*f* likewise shows the plot using method B. In all cases the experimental probability $V(x)$ corresponds closely to the theoretical, indicating that no x-directional influence was acting on the cells. Figs. 4 and 5 show probability $V(x)$ against distance plots for cells moving in the presence of bacteria (chemotaxis) at the

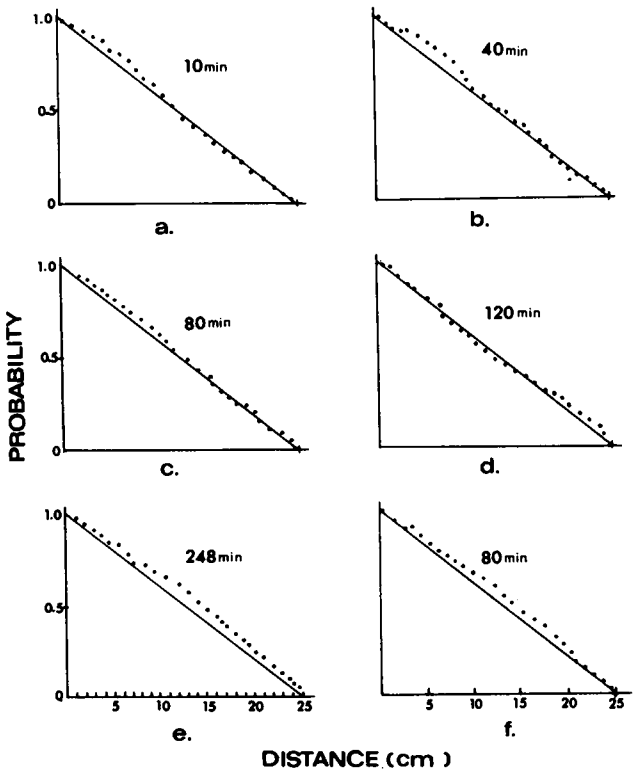

FIGURE 3 (*a–e*) Probability $V(x)$: distance for individual control (neutral) experiments using method A. (*f*) Probability $V(x)$: distance plots for six separate experiments (method B).

indicated times. The increase in probability at given distances indicates that the cells are being attracted towards 0. The maximal response is reached at approximately 90 min after initiation of the experiment and thereafter remains fairly constant until the experiment was terminated at 245 min. The data used to prepare these graphs are presented in the Appendix.

In order to determine the confidence limits of the control (neutral) data the mean and SEM was computed for three selected distances (strips), irrespective of time. For the 5 cm strip, the mean probability $V(x) \pm$ SEM was 0.831 ± 0.0075; 12 cm strip 0.536 ± 0.0123; and for 20 cm strip was 0.222 ± 0.0168. To each mean two standard deviations were added and these values are given in Table I. For any probability value $V(x)$ which is greater than the control mean +2 SD for a given distance we are 95.45% certain that that value does not belong to the control situation; i.e. it represents chemotaxis.

Table I shows the control means plus 2 SDs for these three distances and compares it with the probability values $V(x)$ for both the six separate experiments and the chemotaxis data at various times. It should be noted that the probability $V(x)$ values for the six separate experiments (method B) easily lie within one or two standard deviations

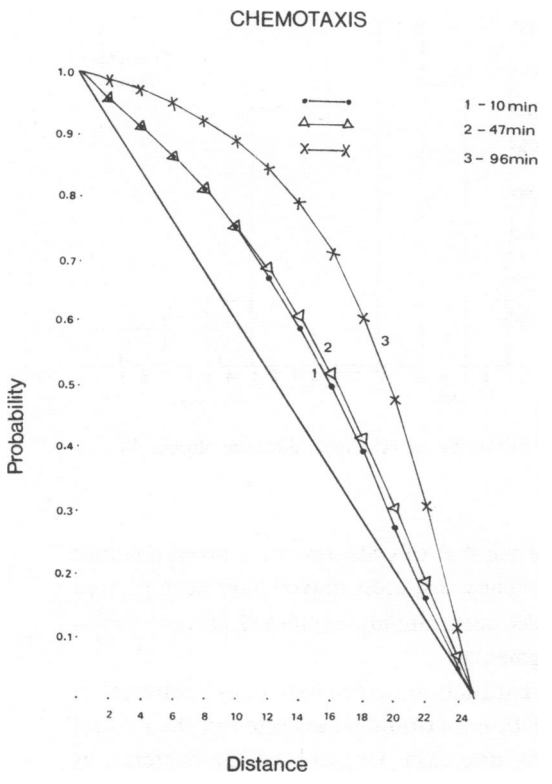

FIGURE 4

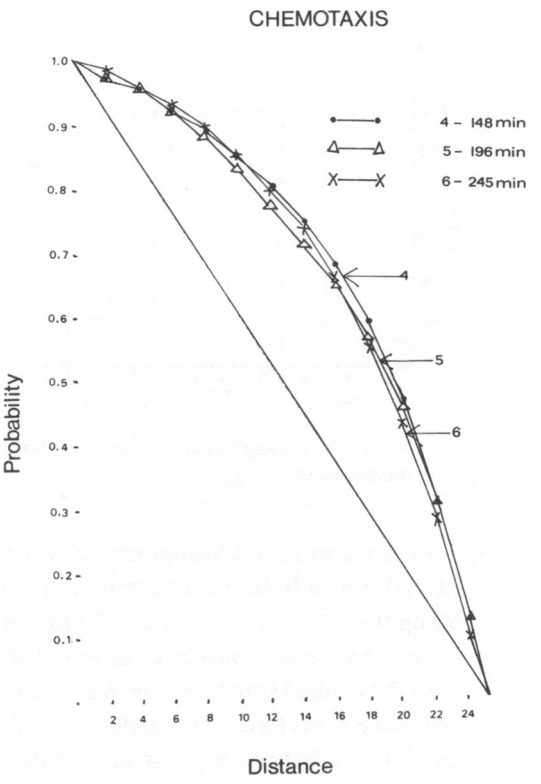

FIGURE 5

FIGURES 4 AND 5 Probability $V(x)$: distance plots at times during chemotaxis.

of values obtained by method A, and consequently one is justified in using method A, which is less time consuming. In the chemotaxis experiments the probability values $V(x)$ are much greater, reflecting attraction of the cells toward the bacteria. The exception is the 10 min value at 20 cm and this suggests that the chemotactic substance has not had time to diffuse to the 20 cm region and influence cell movement.

TABLE I

CONTROL MEANS PLUS 2 SD COMPARED WITH PROBABILITY VALUES

Distance	Probability							
	Control (mean + 2 SD)	6 Separate experiments	Chemotaxis					
			10 min	45 min	96 min	128 min	180 min	248 min
cm								
5	0.865	0.841	0.8867	0.8854	0.9555	0.9358	0.9347	0.9435
12	0.596	0.583	0.6522	0.6641	0.8275	0.7836	0.7512	0.7764
20	0.297	0.258	0.2776	0.2991	0.4724	0.4610	0.4533	0.4267

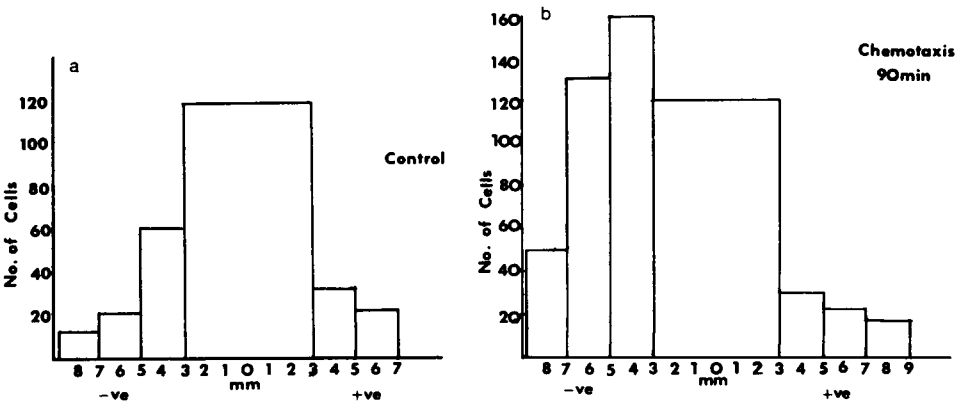

FIGURE 6 Histograms for distance (millimeters) moved by all cells in x direction during 10 frame time span.

Figs. 6*a* and *b* are histograms showing the number of cells moving a given distance ($\pm x$) during a 10 frame time span. For convenience, distances moved have been plotted along the abscissa in groups of 2 mm. As has been already explained all $\pm x$ values <3 mm have been considered as zero displacements.

In the control situation, Fig. 6*a*, it is apparent that approximately equal numbers of cell movements have been made in either direction, as would be expected in the neutral case. Fig. 6*b* shows a progressive shift in the direction, i.e., toward the bacteria, as would be expected in chemotaxis. Fig. 6*a* and 6*b* provide a rough verification that cells are being directionally influenced toward the bacteria.

DISCUSSION

We have applied to a known example of chemotaxis a one-dimensional diffusion process and have shown that it can distinguish chemotactic movements of human granulocytes. In the absence of bacteria (neutral situation) the probability values $V(x)$ approximate the theoretical values expected if no attractive influence is acting on the cells whereas, in the presence of bacteria (chemotaxis), the probability values $V(x)$ are much greater than the control case. The technique outlined has several advantages. Information, expressed as a probability value, can be obtained for cell movements for any point in time and distance for the duration of the experiment. A permanent record of the cell movements is stored on film and these data can be reanalyzed as required for any time interval for the course of the experiment. Negative chemotaxis should also be demonstrable, if present, by this technique.

To reproduce this analytical capability and flexibility using Boyden chambers would not be feasible logistically. Analysis using stochastic differential equations does not share the potential technical difficulties of the Boyden technique. These technical difficulties have been discussed recently and need not be discussed in detail here (11).

In particular, the analytical technique outlined in this paper is not affected by the possibility of a test substance causing enhanced locomotion of cells which in the Boyden technique would increase the probability of a cell finding a "pore" and migrating to the other side of the filter. This might be interpreted as chemotaxis. In this new approach, although the distance moved in unit time would be greater, it would not affect the distribution of $\pm x$ components and the probability $V(x)$ for a given distance would still approximate to the control (neutral) situation. Another advantage of this technique is the low number of cells required for analysis. This is of particular importance, for example, in studying the relationship between leukocytes and tumor cells. Usually, in this situation too few leukocytes can be obtained to use the Boyden technique.

Our initial concern in using method A was that we would be essentially summing the same cell movements and therefore produce a marked bias in the final analysis. Had a bias been introduced the results would most likely not have been as close to the theoretical values (the straight line from 1 to α [Figs. 1 and 3]). Also the probability values obtained for the six separate experiments, where different cells were used, were not significantly different. Lastly, in practice for the control (neutral) situation, it was found that individual cells started, stopped, and restarted within the 20 frame block used for analysis of six 10 frame units. Therefore we feel justified in using method A for although the cells are the same the measured cell movements are different and in taking six 10 frame units close together these are not repetitions of the movements seen in the first 10 frames. The obvious advantages of method A are that only one film is taken and the final analysis is obtained much more quickly. If required, one could also take six 10 frame units over a larger period of time, i.e. 130 frames (17 min 20 s). In this case the probability:distance plot obtained is a statement of what is happening with respect to cell movements over this time interval. However it should be noted that in this latter time interval the chemotactic status may change.

It is interesting to note that the "x" component mean velocity of approximately 15 μm/min does not increase in chemotaxis. This result is in agreement with reports that the actual velocity of cells does not increase in chemotaxis (15, 16).

The reproducibility of the technique for the control data is good (cf. Fig. 3). For the chemotactic experiments the inherent difficulty lies in reproducing exactly the same bacterial system, since we do not know which bacterial products are acting as the chemotactic agents. We have no reason to suspect that the technique of analysis should be any less reproducible for chemotaxis than it is for the controls given reproducible chemotactic gradients. We are presently investigating the sensitivity and reproducibility of this technique using a chemotactic agent, cAMP, which can be readily quantitated. Previous reports on the possible chemotactic effects of cAMP upon neutrophils are contradictory (17–20). Our preliminary results show that we can detect a quantitative difference in chemotactic responses between 10^{-4} M and 10^{-5} M cAMP (Noble, P. B., A. Boyarsky, and S. C. Peterson, in preparation).

We feel that the method presented offers a flexible analytical means of assessing

chemotaxis and should prove useful in the identification of chemotactic substances of importance to many immunologic and physiologic systems.

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APPENDIX

PROBABILITY: CONTROL

Distance	Theoretical	10 min	40 min	80 min	120 min	248 min	80 min (6 exp)
1.0	0.96	0.970	0.967	0.964	0.972	0.967	0.969
2.0	0.92	0.942	0.937	0.930	0.935	0.933	0.939
3.0	0.88	0.912	0.909	0.896	0.892	0.898	0.908
4.0	0.84	0.879	0.881	0.863	0.849	0.864	0.876
5.0	0.80	0.845	0.850	0.828	0.807	0.827	0.841
6.0	0.76	0.809	0.816	0.791	0.762	0.789	0.805
7.0	0.72	0.772	0.775	0.751	0.715	0.752	0.771
8.0_a	0.68	0.732	0.727	0.707	0.670	0.720	0.736
9.0	0.64	0.687	0.675	0.663	0.623	0.689	0.699
10.0	0.60	0.632	0.624	0.621	0.579	0.657	0.660
11.0	0.56	0.575	0.576	0.579	0.541	0.622	0.621
12.0	0.52	0.522	0.530	0.535	0.507	0.586	0.583
13.0	0.48	0.474	0.486	0.490	0.474	0.552	0.548
14.0	0.44	0.427	0.445	0.445	0.441	0.518	0.513
15.0	0.40	0.382	0.407	0.403	0.409	0.482	0.479
16.0	0.36	0.338	0.370	0.363	0.379	0.444	0.444
17.0	0.32	0.297	0.331	0.324	0.351	0.402	0.403
18.0	0.28	0.259	0.286	0.287	0.322	0.358	0.358
19.0	0.24	0.224	0.237	0.249	0.291	0.313	0.310
20.0	0.20	0.186	0.188	0.212	0.256	0.267	0.258
21.0	0.16	0.147	0.145	0.173	0.216	0.220	0.205
22.0	0.12	0.109	0.105	0.132	0.169	0.171	0.151
23.0	0.08	0.072	0.069	0.090	0.115	0.120	0.097
24.0	0.04	0.036	0.035	0.045	0.057	0.061	0.047
25.0	0.00	0.000	0.000	0.000	0.000	0.000	0.000

PROBABILITY: CHEMOTAXIS

Distance	Theoretical	10 min	47 min	96 min	148 min	196 min	241 min
1.0	0.96	0.977	0.978	0.992	0.989	0.990	0.991
2.0	0.92	0.954	0.955	0.984	0.978	0.978	0.981
3.0	0.88	0.931	0.932	0.975	0.965	0.965	0.970
4.0	0.84	0.909	0.909	0.965	0.950	0.950	0.957
5.0	0.80	0.886	0.885	0.955	0.935	0.934	0.943
6.0	0.76	0.863	0.861	0.944	0.920	0.916	0.928
7.0	0.72	0.837	0.836	0.932	0.903	0.896	0.911
8.0	0.68	0.810	0.808	0.917	0.885	0.874	0.892
9.0	0.64	0.781	0.780	0.901	0.867	0.849	0.872
10.0	0.60	0.747	0.751	0.884	0.846	0.824	0.846
11.0	0.56	0.710	0.718	0.863	0.822	0.796	0.820
12.0	0.52	0.671	0.682	0.840	0.797	0.766	0.791
13.0	0.48	0.632	0.644	0.813	0.769	0.736	0.760
14.0	0.44	0.590	0.603	0.783	0.741	0.706	0.727
15.0	0.40	0.547	0.560	0.746	0.709	0.673	0.689
16.0	0.36	0.499	0.514	0.703	0.673	0.639	0.647
17.0	0.32	0.446	0.464	0.655	0.632	0.601	0.600
18.0	0.28	0.391	0.411	0.601	0.583	0.559	0.548
19.0	0.24	0.335	0.355	0.541	0.526	0.510	0.492
20.0	0.20	0.277	0.299	0.472	0.461	0.453	0.426
21.0	0.16	0.216	0.243	0.391	0.385	0.388	0.353
22.0	0.12	0.158	0.186	0.304	0.304	0.313	0.275
23.0	0.08	0.104	0.126	0.209	0.217	0.225	0.188
24.0	0.04	0.051	0.064	0.110	0.118	0.124	0.095
25.0	0.00	0.000	0.000	0.000	0.000	0.000	0.000