Analysis of the Directed and Nondirected Movement of Human Granulocytes: Influence of Temperature and ECHO 9 Virus on *N*-formylmethionylleucylphenylalanine-induced Chemokinesis and Chemotaxis

BURKHARD D. BÜLTMANN and HANS GRULER
Abteilung für Pathologie der Universität Ulm, 7900 Ulm (Donau), Federal Republic of Germany; and Abteilung für Biophysik der Universität Ulm, 7900 Ulm (Donau), Federal Republic of Germany

ABSTRACT The directed movement of human polymorphonuclear leukocytes (PMN) in a plane (Zigmond chamber assay) is described by a statistical model. We demonstrate that (*a*) the movement of a single cell is a superposition of a directed and a random movement, and (*b*) the degree of orientation, P_1, of moving cells in a chemotactic gradient can be determined either by the time average of a single cell or by the average of movement of multiple cells at a fixed time (Ergoden hypothesis). However, an homogeneous cell population is a necessary condition. P_1, which is identical with the McCutcheon index, is derived from the measured angular distribution function of moving cells. The statistical model allows one to distinguish between chemotaxis and chemokinesis. Applying this model to the temperature-dependent changes of cell movement, we found that $P_1 = 0.82$ (37°C) decreased to $P_1 = 0.4$ (22°C). The average speed of moving cells exhibits a very strong temperature-dependent variation from 30 μm/min (37°C) to 5 μm/min (22°C), indicating a different temperature dependence of chemotaxis and chemokinesis. At a fixed temperature (37°C) the stability of the chemotactic gradient can also be checked by the angular distribution function. In addition, this model was applied to investigate the enteric cytopathogenic human orphan, strain 9 (ECHO 9) virus-induced disturbances of cell movement. We found: (*a*) The average speed of cell movement is not affected by the virus. (*b*) The degree of orientation is not affected for virus doses below a critical virus dose, a_0 (virus/PMN = 0.8:1). (*c*) The degree of orientation above this critical value exhibits a time- and virus-dose-dependence. (*d*) At a fixed viral dose, the time-dependent decrease of P_1 is described by an exponential law (virus/PMN = 5:1, the characteristic time is 110 min). (*e*) This characteristic time investigated as a function of viral dose results in a logarithmic law analogous with the Weber-Fechner law. These findings indicate that only chemotactic and not chemokinetic response is disturbed by ECHO 9 virus.

The ability of some cells or primitive organisms to direct their movement along a chemical gradient seems to be a general phenomenon (1). Lower organisms, e.g., bacteria or slime molds, exhibit movements directed to nutrients. Following infection or other damage of mammalian tissue, chemotactic factors are released and induce movement of polymorphonuclear leukocytes (PMN) to the site of injury. During this process these cells can orient their locomotion according to a chemical gradient, i.e., exhibit chemotaxis (2). This chemotactic reactivity has been tested in vitro using different assays. One of them was designed by Zigmond (2) and will be employed in our investigations. Beside these different in vitro assays, there exist some statistical models of cell movement that are based on the diffusion equation (3–6). The purpose of this paper is to demonstrate a general mathematical model of directed cell movement, and to show the relationship between directed and nondirected movement, as well as the relationship between the movement of an individual cell and that of a population of

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 JUNE 1983 1708–1716

cells. Our model is a mathematical description of cell or particle movement and thus, necessarily, is not restricted to human PMN. The practical importance of our investigations lies in a detailed analysis of the experimental conditions of the Zigmond chamber assay to demonstrate that preincubation of human PMN with enteric cytopathogenic human orphan virus, type 9, strain A. Barty (ECHO 9 A.B.) results in disturbances of cellular response to chemoattractants (zymosan-activated serum, *N*-formylmethionylleucylphenylalanine). This technique, however, has certain limitations regarding the discrimination between chemokinesis and chemotaxis (8). Therefore in the present investigations, the Zigmond chamber assay and our mathematical model of cell movement was applied for further analysis of this virus-induced phenomenon. We will show that only chemotactic but not chemokinetic reactivity of virus-treated PMN will be inhibited.

analysis of this virus-induced phenomenon. We will show that only chemotactic but not chemokinetic reactivity of virus-treated PMN will be inhibited.

MATERIALS AND METHODS

Granulocytes

Leukocytes were separated from heparinized venous blood of healthy human blood donors by means of dextran sedimentation or Ficoll-Hypaque density gradient as previously described (8). After osmotic lysis of the remaining red blood cells and readjustment of osmotic strength, the leukocytes were washed three times, resuspended in Hanks' solution supplemented with 1% gelatine (Hanks' gel), and adjusted to 5×10^6 PMN/ml as stock suspension.

Virus

ECHO 9 A. B. was originally derived from a patient with aseptic meningitis (10). As previously reported, this strain was propagated in primary cell cultures of trypsinized kidney tissue from African green monkeys (Flow Laboratories, Inc., McLean, VA); for infectivity titrations (plaque-forming units), a continuous cell line derived from African green monkey renal tissue was used (7).

To study the interaction of ECHO 9 A. B. virus with granulocyte function, 2×10^6 PMN/ml were preincubated (t_p) with different high doses of virus (2×10^6, 1×10^7, 4×10^7, and 1×10^8 pfu) for 15 min at 37°C in a shaking water bath. Some preparations of 2×10^6 PMN/ml were preincubated with 1×10^7 pfu ECHO 9 A. B. virus for 60 min at 37°C. Subsequently, the cells were washed 3 times and resuspended to the original volume. The virus suspension was sonified for 5 s before incubation with PMN.

Chemotactic Factor

The chemotactic factor used was *N*-formylmethionylleucylphenylalanine (FMLP) supplied by Vega-Biochemicals (Div. of Vega Biotechnologies, Inc., Tucson, AZ). Stock solutions were prepared with dimethyl sulfoxide and stored at −20°C. Assays of FMLP were carried out in Hanks' gel in concentrations of 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M.

Chemotaxis Assay

The chemotactic chambers were set up as described by Zigmond (2). Briefly, 50 μl of the cell suspension were pipetted on a round (22 mm ϕ) No. 1 glass coverslip. The cells attached to the glass surface to form a monolayer during incubation for 15 min in a 37°C humid incubator gassed with 5% CO_2. The coverslip with adherent PMN was rinsed twice with Hanks' gel, then inverted, placed on the Zigmond chamber, and secured with brass clips. Excess buffer was removed by gentle suction; the wells were filled with 100 μl of FMLP in one well, whereas the other contained Hanks' gel only. Both wells were sealed by paraffin wax to avoid loss of medium by evaporation. The loaded Zigmond chamber was placed on the heated (37°C) object stage of a Zeiss photomicroscope equipped with an interference optical system (×40).

Mathematical Model of Directed Cell Movement

MODEL OF INDIVIDUAL CELL MOVEMENT: We have the following problem: A granulocyte adhering to a plane surface has the intrinsic possibility of motility. This is demonstrated by the fact that irregular cytoplasmic propulsions or protuberances are always present without change in the original adhering position (Fig. 1 *a*). Exposure of the cell to a chemotactic stimulus results in active movement accompanied by characteristic cell shape changes and by change of the starting position (Fig. 1, *b* and *c*). The movement of the center of the granulocyte mass (*S*) in a plane (Fig. 2) can be described by the following vector:

$$\mathbf{v} = \hat{\mathbf{x}} \cdot V_c \cdot \cos\varphi + \hat{\mathbf{y}} \cdot V_c \cdot \sin\varphi. \tag{1}$$

$\hat{\mathbf{x}}$ and $\hat{\mathbf{y}}$ are cartesian unit vectors in the plane. The speed of the granulocyte is described by the track velocity V_c. The direction of movement is described by the angle φ. The path of the migrating cell is described by the time dependence of the angle $\varphi(t)$. It is assumed that V_c is constant in time at standardized experimental conditions. At time zero, the cell is at the starting point. After the time t, the cell migrated in both x and y directions.

$$\mathbf{x} = \int_0^t V_c \cdot \cos\varphi(t) \cdot dt, \tag{2}$$

$$\mathbf{y} = \int_0^t V_c \cdot \sin\varphi(t) \cdot dt. \tag{3}$$

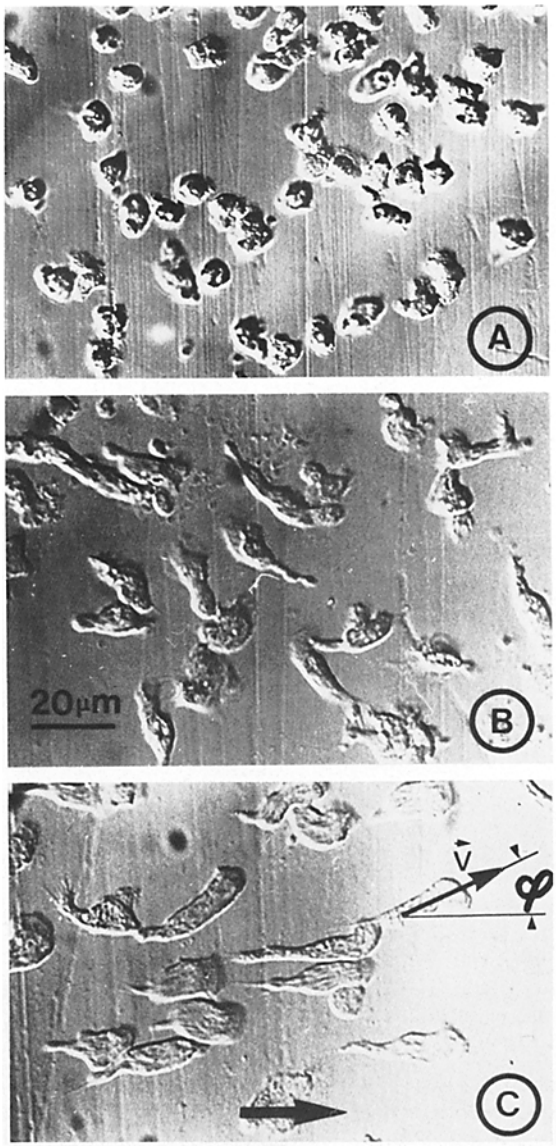

FIGURE 1 Cytological changes of granulocytes in a Zigmond chamber. (*a*) Random mobility of cells not exposed to chemotactic agents. (*b*) Chemokinetic movement induced by a homogeneous environment of 10^{-8} M FMLP. (*c*) Chemotactic response of PMN exposed to a chemotactic gradient of 10^{-8} M FMLP/mm (fat arrow). Angle φ is defined by vectors of chemotactic gradient and velocity **v** of granulocyte.

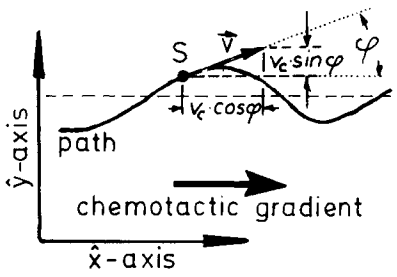

FIGURE 2 Schematic representation of the movement of a single granulocyte (heavy line) in the $\hat{x}$-$\hat{y}$ plane. At a given time the position of the granulocyte (center of the mass) is S, and the corresponding velocity, $\mathbf{v}$. The angle φ is defined by the vectors of the track velocity and the chemotactic gradient. The velocity parallel and perpendicular to the chemotactic gradient is $v_c \cdot \cos\varphi$ and $v_c \cdot \sin\varphi$, respectively.

These equations can be rewritten as

$$\mathbf{x} = V_c \cdot t \cdot \langle \cos\varphi(t) \rangle_t, \tag{4}$$

$$\mathbf{y} = V_c \cdot t \cdot \langle \sin\varphi(t) \rangle_t. \tag{5}$$

$\langle \cos\varphi \rangle_t$ and $\langle \sin\varphi \rangle_t$ are the time average of $\cos\varphi(t)$ and $\sin\varphi(t)$.

$$\langle \cos\varphi \rangle_t = \frac{1}{t} \int_0^t \cos\varphi(t) \cdot dt, \tag{6}$$

$$\langle \sin\varphi \rangle_t = \frac{1}{t} \int_0^t \sin\varphi(t) \cdot dt. \tag{7}$$

For a nondirected movement, $\langle \cos\varphi \rangle_t$ and $\langle \sin\varphi \rangle_t$ are zero. This means that the migrating cell moves in a random fashion around the starting position. This type of movement is referred to as chemokinesis or random mobility (8, 9) of the granulocyte (Fig. 1 *b*). In our case the random mobility can be characterized by the track velocity, $V_c \cdot V_c$ is dependent on environmental conditions such as the strength of chemotactic agent, pH, and temperature (8, 12). In a directed movement, $\langle \cos\varphi \rangle_t$ and $\langle \sin\varphi \rangle_t$ are not equal to zero owing to cell orientation accompanied by characteristic changes of cell shape (Fig. 1 *c*). A preferred direction can be achieved by introducing a chemical gradient of a chemotactic material into the experimental system (Fig. 2). This kind of directed cell movement is referred to as chemotaxis (3, 8, 9). If this preferred direction is parallel to the $\hat{x}$ direction of the plane (chemotactic gradient), then

$$\langle \cos\varphi \rangle_t \neq 0, \tag{8}$$

$$\langle \sin\varphi \rangle_t = 0. \tag{9}$$

Since $V_c \cdot \langle \cos\varphi \rangle_t$ describes the strength of the directed cell movement, we will use $\langle \cos\varphi \rangle_t$ to refer to the degree of cell orientation P_1. This definition is used in analogy with a physical system describing the orientation of molecules in a field (e.g., the orientation of elementary magnets in a magnetic field or the orientation of molecular electric dipoles in an electric field).

$$P_1 = \langle \cos\varphi \rangle_t = \frac{1}{t} \int_0^t \cos\varphi(t) \cdot dt \tag{10}$$

The maximal degree of orientation can be 1. In this situation the cell moves in a straight line parallel to the preferred direction. In the case of $0 < P_1 < 1$, the cell moves in a snakelike path in the direction of the chemotactic gradient, as shown in Fig. 2. The average speed in the direction of the chemotactic gradient, $V_\parallel$, defines the biological function (chemotaxis):

$$V_\parallel = V_c \cdot P_1. \tag{11}$$

Based on this general mathematical description of cell movement, it is possible to measure quantitatively the chemotactic response of an individual granulocyte in a Zigmond chamber exposed to a chemotactic gradient. The speed V_c and the degree of orientation P_1 can be evaluated from a series of pictures taken consecutively and photographed during a period of time after the chemotactic gradient has been stabilized (Fig. 3).

$$V_c = \frac{1}{N_0 - 1} \sum_{i=1}^{N_0} \frac{\sqrt{\Delta x_i^2 + \Delta y_i^2}}{\Delta t}. \tag{12}$$

The degree of orientation is

$$P_1 = \frac{1}{N_0 - 1} \sum_{i=1}^{N_0} \frac{\Delta x_i}{\sqrt{\Delta x_i^2 + \Delta y_i^2}}. \tag{13}$$

N_0 is the total number of pictures.

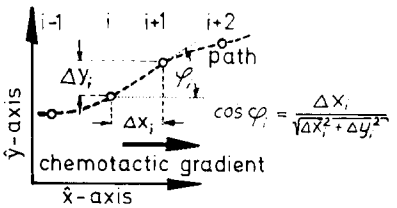

FIGURE 3 Schematic representation of a single cell on time-lapse film ($\Delta t = 15$ s). Circles represent the position of a granulocyte at different times $t = \Delta t \cdot (\ldots, i-1, i, i+1, \ldots)$. The actual angle φ_i is determined by pairs of two consecutive points $(i, i+1)$.

ANALYSIS OF A POPULATION OF CELLS IN A ZIGMOND CHAMBER: As Valone (9) pointed out, there are two general categories of methods for assessing the movement of PMN: observation of individual cell motion or measurement of the migration of a cell population in an experimental system. The last method determines only the average degree of cell orientation at a given time during the experiment after a chemotactic gradient has been stabilized. Usually, the percentage of oriented cells describes the degree of chemotactic response (2, 13). A more accurate analysis can be achieved using the Ergoden hypothesis of Boltzmann (14). It relates the cell movement of an individual cell at different times (time average) to the cell orientation of a population of cells (pop) at a fixed time (average of cell population).

$$\langle \cos\varphi \rangle_t = \langle \cos\varphi \rangle_{pop}. \tag{14}$$

The degree of orientation of a single cell $\langle \cos\varphi \rangle_t$ is already discussed in the previous section (see Eq. 6 and 7). $\langle \cos\varphi \rangle_{pop}$ is described by the following formula:

$$\langle \cos\varphi \rangle_{pop} = \frac{1}{N_0} \sum_{i=1}^{N_0} \cos\varphi_i. \tag{15}$$

N_0 is the total number of cells in micrographs taken at fixed times during the experiment. φ_i represents the angle of the ith cell. In the further discussion we will use the symbol P_1 for the degree of orientation, which is identical with $\langle \cos\varphi \rangle_{pop}$. The migrating cells show characteristic shape changes recognizable as cell elongation in the direction of cell motion (Fig. 1 *c*). Therefore the angles φ_i can be determined, and one or more pictures of a population of cells (~100) can be used to evaluate the degree of orientation, P_1, as index for the chemotactic response. If a histogram of the angular distribution function, $N(\varphi)$, is constructed for a population of moving cells, the degree of orientation P_1 can be calculated as

$$P_1 = \frac{1}{N_0} \sum N(\varphi) \cdot \cos\varphi. \tag{16}$$

In an ergodic system the mean value of the same parameter measured on one particle over a long period of time is identical with the mean value of the same parameter investigated by a population of particles at a fixed time (14). Therefore, we do not need to distinguish between the statistical behavior of an individual PMN and that of a cell population. Consequently, $V_\parallel$ can be obtained by determination of V_c and P_1. Therefore Eq. 11 can be used in different variations:

$$\langle V_\parallel \rangle_{pop} = \langle V_c \rangle_{pop} \cdot P_{1pop},$$
$$\langle V_\parallel \rangle_{time} = \langle V_c \rangle_{pop} \cdot P_{1pop}, \tag{11a}$$

$$\langle V_\parallel \rangle_{pop} = \langle V_c \rangle_{time} \cdot P_{1pop},$$
$$\langle V_\parallel \rangle_{time} = \langle V_c \rangle_{time} \cdot P_{1pop}, \tag{11b}$$

$$\langle V_\parallel \rangle_{pop} = \langle V_c \rangle_{pop} \cdot P_{1time},$$
$$\langle V_\parallel \rangle_{time} = \langle V_c \rangle_{pop} \cdot P_{1time}, \tag{11c}$$

$$\langle V_\parallel \rangle_{pop} = \langle V_c \rangle_{time} \cdot P_{1time}, \text{ and}$$
$$\langle V_\parallel \rangle_{time} = \langle V_c \rangle_{time} \cdot P_{1time}. \tag{11d}$$

The index "pop" indicates that the average value characterizes a population of PMN. The mean value with the index "time" describes the statistical behavior of a single cell over a long period of time.

RESULTS

Comparison between Single Cell Movement and the Behavior of a Population of PMN in a Chemotactic Gradient

For investigation of the track velocity, V_c, and the degree of orientation, P_{1time}, of individual moving PMN (n = 348 cells/10 experiments), the granulocytes were exposed to 10^{-8} M FMLP/mm. In the established chemotactic gradient (15–20 min after loading the Zigmond chamber) the cells (20–40 PMN/time and experiment) were followed by a series of single micrographs taken every 20 s for 5 min. All cells in the high power microscopic field were investigated independent of their initial shapes.

As shown in Fig. 4, the real path of an individual granulocyte results in a snakelike movement parallel to the chemotactic gradient. The track velocity, V_c, of this granulocyte was 30 μm/min. The average track velocity of the other granulocytes was 30 ± 5 μm/min. The distribution function, $N(V_c)$, resulted in a single, broadly distributed gaussian curve (σ = 5 μm/min). This indicates that the track velocity fluctuates during the measured time but we have no indication from our experimental data that subpopulations of moving cells are present.

From the same PMN used for the determination of the track velocity, the degree of orientation, P_{1time}, was derived. A small period of the snakelike path of the granulocyte shown in Fig. 4 is schematically drawn in Fig. 3. The local orientation of the PMN during this path at different times is described by the angle φ_i (see Fig. 4). Therefore we can determine the angular distribution function $N(\varphi)$ of such a snakelike path, which is shown on the right side of Fig. 4. As expected for a snakelike path, the distribution, $N(\varphi)$, exhibits a maximum at 0°. However, the movement of the cell is not a straight line parallel to the chemotactic gradient. Therefore the distribution function $N(\varphi)$ reveals variations of cell orientation at different times. The degree of orientation of this snakelike path (left side of Fig. 4) is P_{1time} = 0.8 according to Eq. 13. The average degree of orientation, P_{1time}, of the other moving single cells is 0.8 with a standard deviation of ±0.05. Analysis of the distribution function of $N(P_{1time})$ reveals a narrow-based gaussian curve (σ = 0.05) without evidence for subpopulations.

Next, the degree of orientation of a population of cells, P_{1pop}, at a fixed time was calculated. For this purpose the angular distribution function $N(\varphi)$ of 80–100 PMN was determined from 4–6 photographic pictures/time taken at t_m = 3, 15, 30, and 45 min after loading the Zigmond chamber. Fig. 5*a* summarizes the angular distribution of a cell population of a representative experiment in which granulocytes have been exposed to a 10^{-8} M/mm gradient of FMLP for 45 min. The distribution functions, $N(\varphi)$, peak around 0°. This indicates that most cells are oriented parallel to the chemotactic gradient

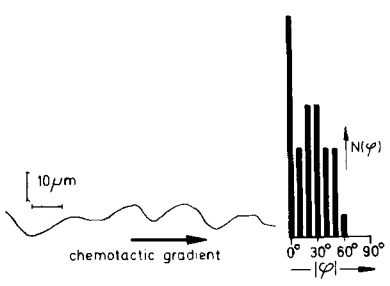

FIGURE 4 *Left side:* Real path of a single cell in a chemotactic gradient (10^{-8} M FMLP/1 mm at 37°C) over 2 min. *Right side:* Angular distribution function $N(\varphi)$ of the snakelike movement of the same granulocyte (data evaluation, see Fig. 3).

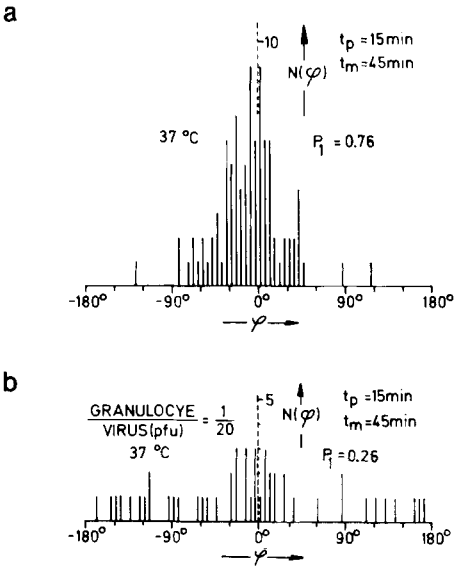

FIGURE 5 Angular distribution function $N(\varphi)$ of a population of PMN in a chemotactic gradient (10^{-8} M FMLP/mm) at a given time (t_m = 45 min). (*a*) Control granulocytes. (*b*) Virus-treated PMN (granulocyte/virus = 1:20).

(Fig. 1*c*). The degree of orientation for this experiment was P_{1pop} = 0.85. A series of further experiments (n = 5) under identical conditions revealed that P_{1pop} was reproducible within only small statistical variations with an average of P_{1pop} = 0.82 ± 0.05 for 520 PMN (t_m = 45 min). The distribution function, $N(P_{1pop})$, is described by a narrow-based (σ = 0.05) gaussian curve without evidence for subpopulations. As expected according to Eq. 14, the degree of orientation, P_{1pop} determined for a population of PMN is identical within the same statistical range with the degree of orientation, P_{1time}, of an individual moving granulocyte.

Influence of Temperature on FMLP-induced Cell Movement

As already mentioned, various factors influence cell movement and orientation (8, 9). Relatively little information is available from the literature about the effect of temperature (12). Therefore, we exposed PMN to 10^{-8} M FMLP/mm at 37°C and at room temperature (22°C) and took micrographs of 80–120 PMN/time at 3, 15, 30, and 45 min after loading the Zigmond chamber. The results are shown in Fig. 6. During the first 10 min after the beginning of the experiments (n = 3/temperature), the chemotactic gradient is not established (dashed line in Fig. 6) according to Zigmond (2). As expected, P_{1pop} scattered around 0° for both temperatures. After the chemotactic gradient stabilized, P_{1pop}, exhibited temperature-dependent marked variations: (*a*) Cells (n = 285 PMN) exposed to FMLP at 37°C for 45 min approach the saturation value of P_{1pop} = 0.82 ± 0.05. (*b*) For PMN (n = 326) exposed to the same chemotactic gradient at 22°C for 45 min, the saturation value P_{1pop} reached only 0.4 ± 0.05. The angular distribution function, $N(\varphi)$, and the distribution function, $N(P_i)$ for both temperatures revealed a narrow-based gaussian curve (σ = 0.05), respectively, without evidence for subpopulations.

In three further experiments the track velocity, V_c, of PMN (n = 120/temperature) was investigated by a series of single micrographs taken every 20 s for 5 min in the established chemotactic gradient (t_m = 35–45 min after loading the Zig-

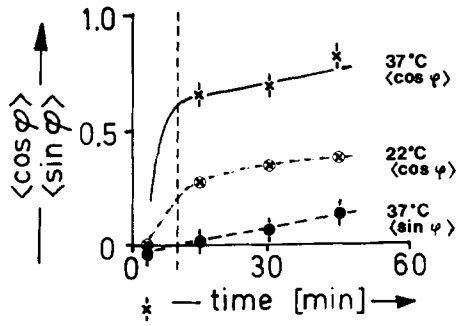

FIGURE 6 The degree of orientation P_1, ⟨cosφ⟩, and the average value of sinφ, ⟨sinφ⟩, of oriented PMN in a chemotactic gradient (10^{-8} M FMLP/mm) at 37°C and P_1 at 22°C as a function of time.

mond chamber). The average track velocity $V_{c37°C}$ was 30 ± 2.8 μm/min, whereas $V_{c22°C}$ reached only 5 ± 1.5 μm/min. The distribution functions $N(V_{c37°C})$ and $N(V_{c22°C})$ disclosed a single gaussian curve respectively, without indication of subpopulations.

From the same cells used for the determination of the track velocity, V_c, the degree of orientation, P_{1time}, was derived. For both temperatures, the average values of $P_{1\,time\,37°C} = 0.8$ and $P_{1\,time\,22°C} = 0.39$ equal the experimental data of P_{1pop} at 37°C and 22°C within the same statistical range (±0.05).

Influence of ECHO 9 A. B. Virus on FMLP-induced Cell Movement

On the basis of our recent observations of ECHO 9 A. B. virus-induced diminished cellular chemotactic response of PMN in the Boyden chamber (7), we would now like to discriminate between disturbances of chemotactic and/or chemokinetic behavior of granulocytes. This can be done by evaluating the data of Eq. 11. We determined the degree of orientation, P_{1pop}, of virus-treated cells at different times at 37°C.

In pilot experiments (n = 3) the angular distribution functions $N(\varphi)$ of a population (n = 80–100 cells) of control granulocytes and virus-treated PMN (granulocyte/virus = 1:20, t_p = 15 min) both exposed to 10^{-8} M FMLP/mm for t_m = 45 min have been evaluated. As already shown in Fig. 5*a*, the distribution functions $N(\varphi)$ of control granulocytes peak around 0°. This indicates that most of the cells are oriented parallel to the chemotactic gradient (Fig. 1*c*). The analogous distribution of virus-treated PMN has a less pronounced peak at an angle ~0° (Fig. 5*b*) exhibiting a cell orientation similar to Fig. 1*b*. Using Eq. 17 for these experiments we obtained a degree of orientation for control granulocytes of P_{1pop} = 0.76 ± 0.05 and of P_{1pop} = 0.26 ± 0.05 for virus-treated cells. The track velocity, V_c, derived from a series of micrographs taken every 20 s over 5 min (20–30 min after start of the experiment in the Zigmond chamber), was also measured for both experimental assays. The average track velocity of n = 270 control granulocytes was 30 ± 4.8 μm/min, and for virus-treated PMN (n = 236) 29.6 ± 3.2 μm/min. The distribution function, $N(V_c)$, for both experimental groups disclosed a single gaussian curve without evidence for subpopulations.

In the following experiments (n = 5/viral dose) the influence of viral dose on the degree of orientation of FMLP-induced cell orientation was investigated. Granulocytes were preincubated with different doses of ECHO 9 A. B. virus (granulocyte/virus ratio ranging from 1:1 to 1:50) for t_p = 15 min and then exposed to a chemotactic gradient (10^{-8} M FMLP/mm). Photographs of cell populations (80–120 PMN/time and experiment) were taken t_m = 3, 15, 30, and 45 min after starting the chemotaxis assay. As shown in Fig. 7 the degree of orientation, P_{1pop}, declined in time with rise in the dose of virus from P_1 = 0.8 ± 0.05 of control cells to P_1 = 0.15 ± 0.05 of PMN preincubated with virus (granulocyte/virus = 1:50) 45 min after starting the experiment in the Zigmond chamber. Fig. 8*a* summarizes the viral dose-dependent changes of the degree of orientation at a fixed time t_p = 15 min, t_m = 45 min. Again, a viral dose-dependent decline of the degree of orientation, P_{1pop}, occurs, indicating a change in cellular behavior from chemotactic to chemokinetic response (random mobility).

Next, the degree of orientation, P_{1pop}, is investigated as a

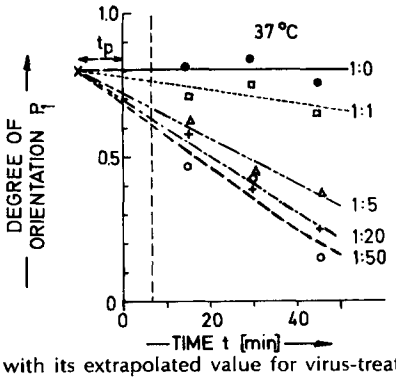

FIGURE 7 Influence of time and viral dose on the degree of orientation, P_1, of virus-treated PMN exposed to 10^{-8} M FMLP/mm. The preincubation time, t_p, is 15 min. The vertical dashed line describes the end of the phase of building up the chemotactic gradient. ●, control granulocytes with its extrapolated value for virus-treated PMN at the time $-t_p$ represented by the point, X, granulocyte/virus ratio: □, 1:1; △, 1:5; +, 1:20; ○, 1:50.

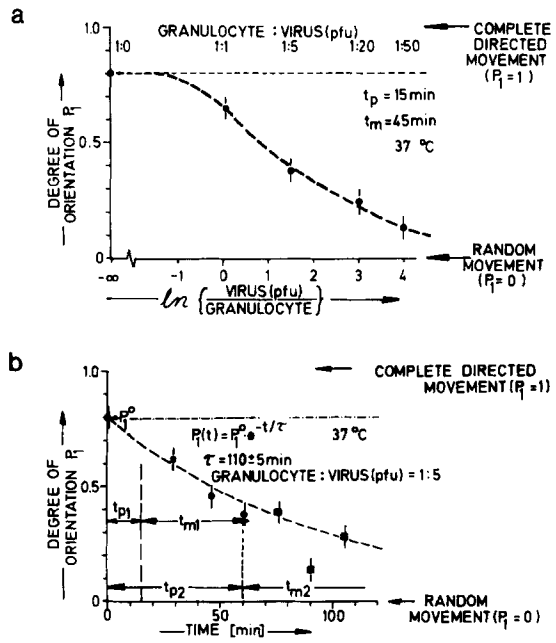

FIGURE 8 (*a*) Influence of viral dose on the degree of orientation, P_1, at a fixed time (t_p = 15 min, t_m = 30 min). In the diagram the natural logarithm (ln) of the virus/granulocyte ratio is used. The horizontal dashed line represents the degree of orientation of control granulocytes. (*b*) Influence of time on the degree of orientation, P_1, at a given viral dose of PMN exposed to 10^{-8} M FMLP/mm. (—·—): degree of orientation of control granulocytes. (●): degree of orientation of virus-treated PMN (preincubation time t_{p1} = 15 min; t_{m1} = 45 min). (■): degree of orientation of virus-treated PMN (t_{p2} = 60 min; t_{m2} = 45 min). The dashed line represents an exponential decline of the degree of orientation of PMN with time of virus interaction with a characteristic decay time τ = 110 ± 5 min.

function of time at a given viral dose. Therefore, PMN were preincubated for t_{p1} = 15 min (n = four experiments) and t_{p2} = 60 min (n = four experiments) with ECHO 9 A. B. virus (granulocyte/virus = 1:5) and then exposed to a chemotactic gradient (10^{-8} M FMLP/mm). The degree of orientation, P_{1pop}, of both cell preparations (n = 786 PMN) before virus-treatment is P_1 = 0.8 ± 0.05 (Fig. 8 *b*), which is the value of control cells, P_1^0. The degree of orientation for a population of PMN (n = 396) preincubated for 15 min (t_{p1}) was measured for 45 min in the Zigmond chamber (t_{m1}). In accordance, t_{m2} describes the degree of orientation of PMN (n = 390) preincubated for 60 min (t_{p2}). The degree of orientation decreases with increasing duration of virus-PMN interaction and can be described by an exponential law:

$$P_1(t) = P_1^0 \cdot e^{-t/\tau}. \quad (17)$$

The exponential function (dashed line in Fig. 8 *b*) is obtained by a "least square fit" procedure with a characteristic decay time, τ, of 110 ± 5 min. This means that the degree of orientation (chemotaxis) is divided by a half-life period of 72 ± 4 min ($t_{1/2} = \tau \ln 2$).

So far, we analyzed the data at a fixed time and at a fixed virus dose. However, it is possible to analyze the data in such a way that the results lead to a better interpretation of the virus-granulocyte interaction. Therefore, the characteristic time, τ, was investigated as a function of virus dose, a. The results are shown in Fig. 9. In this special plot, the data follow a straight line described by

$$\left(\frac{\tau_0}{\tau}\right)^2 = \ln\left(\frac{a}{a_0}\right). \quad (18)$$

The slope of the straight line represents τ_0 which has a value of 2.55 ± 0.2 h. The intersection of the straight line with the abscissa defines a_0. We found for this critical viral dose 0.8 ± 0.1 pfu/PMN.

These data again confirm that a change in cellular behavior occurs above the critical virus dose a_0 resulting in a disturbance of chemotactic but not chemokinetic response.

DISCUSSION

Our experiments have yielded results in five areas of investigation, which are discussed below.

Ergoden Hypothesis

On the basis of our results we have demonstrated that the degree of orientation, P_{1time}, of one moving granulocyte (time average) equals that of a population of cells, P_{1pop} (average of a cell population). This finding confirms the Ergoden hypothesis that was originally formulated by Boltzmann (14) for moving molecules of a homogeneous population. To be certain that one is dealing with a homogeneous cell population, it is still necessary to compare individual cells or small groups of cells with populations of cells. Additional information about the homogeneity of cell populations is in general available from analysis of the distribution functions of different experimental data. For example, analysis of the angular distribution function, $N(\varphi)$, of control PMN exposed to the chemotactic gradient revealed a single gaussian curve without evidence for subpopulations (Fig. 5 *a*). Therefore we conclude that granulocytes moving in a chemotactic gradient behave as an ergodic system. This is of practical importance: Statistical predictions regarding the directed cell movement can be made for an individual cell even when the data have been gathered from a population of cells.

Analysis of the angular distribution function, $N(\varphi)$, of the virus-treated PMN in Fig. 5 *b* reveals four items of information:

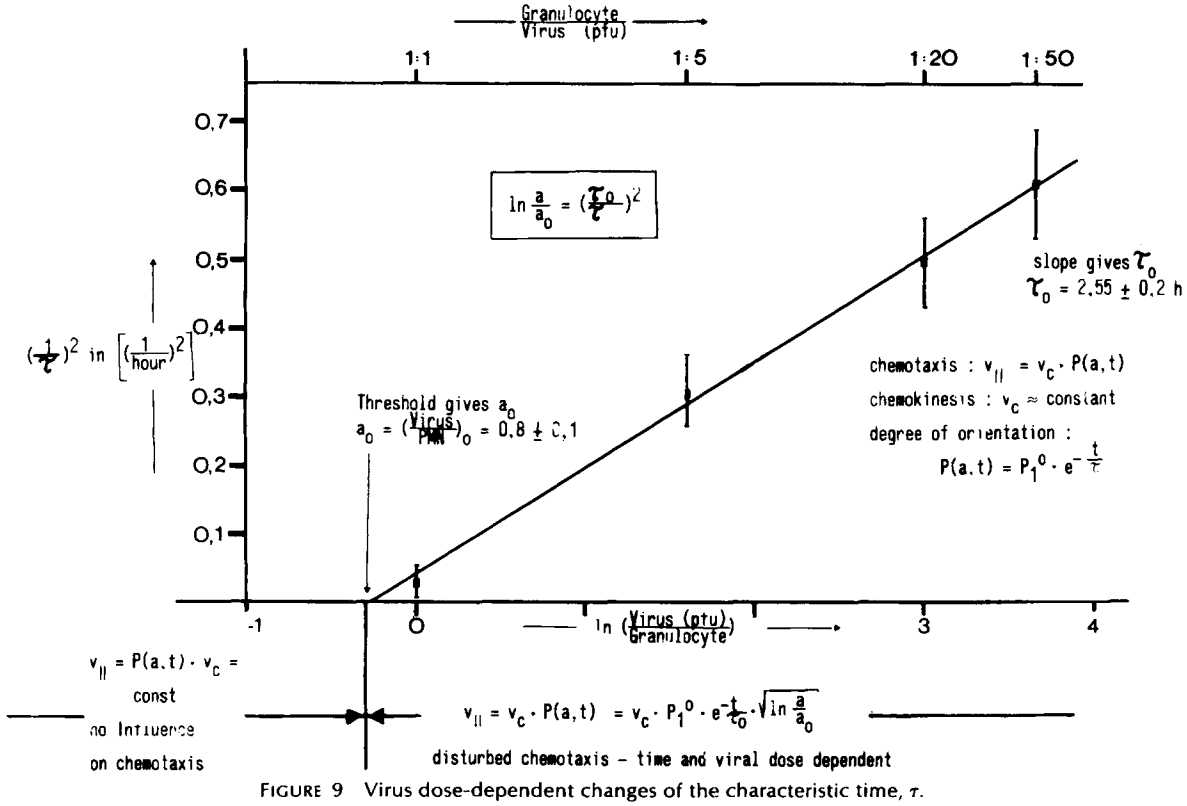

FIGURE 9 Virus dose-dependent changes of the characteristic time, τ.

(*a*) $N(\varphi)$ discloses two types of distribution functions: one peaks around 0°, as we found for control granulocytes, whereas the other population is broadly distributed in the ±180° sector. This indicates that we are dealing with an inhomogeneous cell population. (*b*) The percentage of both cell populations can be calculated: about one-third of the virus-treated PMN are in the ordered state like control granulocytes, whereas the remainder of the virus-treated PMN shows virus-induced disorder for this special experimental condition. (*c*) If we analyze P_{1pop} of the first population (ordered state) of Fig. 5 *b*, we obtain a P_1 degree of orientation of ~0.8, whereas the degree of orientation, P_{1pop}, of the other population is zero. The average degree of orientation of the whole cell population in Fig. 5 *b* is $P_1 = 0.26$. This demonstrates that Eq. 14 does not hold for this experimental condition. Therefore we can not make any statistical prediction for a single cell if the data are gathered from the whole cell population. However, statistical predictions for the whole cell populations are permitted. (*d*) From the results of Fig. 5 *b* we must conclude that according to Eq. 14 the Ergoden hypothesis does not hold for this experimental condition. However, we know from our data of Figs. 8 and 9 that the degree of orientation of virus-treated PMN declines to zero with a characteristic time, τ (τ_0 ~2.5 h). In this virus-induced final state of PMN, again, we are dealing with a homogeneous cell population, and we are allowed to apply the Ergoden hypothesis.

Thus, application of the above mentioned criteria to the analysis of different experimental conditions, e.g., viral dose or time of virus interaction with PMN, permits the identification of cell subpopulations including their percentages, and also the investigation and description of time- or dose-dependent functional changes of target cells.

Degree of Orientation

The definition of the degree of orientation of an individual granulocyte or of a population of cells is given by the mathematical system itself. McCutcheon (15) characterized the oriented cell movement also by the system itself: he compared the distance D parallel to the chemotactic gradient with the actual path length L of the moving granulocyte (Fig. 2). The McCutcheon index D/L is identical to the degree of orientation of our model. This agreement can be confirmed by the following analysis. The real path length L can be divided in small path lengths Δs:

$$\Delta s = v_c \cdot \Delta t. \tag{19}$$

The actual path length is then

$$L = N_0 \cdot \Delta s. \tag{20}$$

The projection of the small path length Δs onto the direction of the chemotactic gradient Δs_p is

$$D = N_0 \cdot \Delta s_p = \sum \Delta s \cdot \cos\varphi. \tag{21}$$

This equation can be rewritten using Eq. 20:

$$D = L \cdot \langle \cos\varphi \rangle, \tag{22}$$

QED.

The degree of orientation obtained by analyzing a single granulocyte is 0.85; the McCutcheon index of the same cell is 0.84; the degree of orientation of the population of cells is 0.85. As expected, the McCutcheon index is in accordance with the degree of orientation. Nossal and Zigmond (16) introduced the chemotropism index. Their description is in accordance with our Eq. 16.

Another parameter for estimating chemotactic reactivity of granulocytes that is mainly used in the literature is the determination of the percentage of oriented cells in the chemotactic gradient. For example, Zigmond (2) counted all granulocytes oriented in a ±90° sector around the chemotactic gradient divided by the total number of cells. This seems representative because 60% of the PMN are oriented within ±30° if a system is chosen with 0.85 degree of orientation. In this parameter some oriented cells are excluded, resulting in a relatively high percentage of oriented PMN. In contrast we counted all cells in a ±180° sector. Zigmond (2) obtained for one set of experimental conditions a percentage of oriented cells of 97%. We analyzed her results in retrospect with our method and arrived at a degree of orientation of 0.85. Furthermore, this comparison shows that the percentage of oriented cells is not equal to the McCutcheon index or the degree of orientation. This is mainly because the definition of the percentage of oriented PMN used by Zigmond and others (2, 8, 13, 17, 18) is not defined by the system itself.

Determination of Local Chemotactic Gradient

The degree of orientation is derived from the angular distribution function $N(\varphi)$. However, $N(\varphi)$ yields much more information about the system. Therefore, it is possible to examine whether the direction of the local chemotactic gradient is in accordance with the direction of the macroscopically applied chemotactic gradient (mean gradient). The directions of the local and the mean gradient are equal if the angular distribution function $N(\varphi)$ is symmetrical around zero (Fig. 4). This situation was assumed in Eqs. 8 and 9, and leads to zero for the average of $\sin\varphi$. However, under experimental conditions there may be small deviations in the local direction of the chemotactic gradient in comparison with the mean direction. In this case the average of $\sin\varphi$ is unequal to zero. This indicates that the evaluation of an experiment should only be performed if $\langle \sin\varphi \rangle$ is zero or less than the statistical error that is in the range of 0.05. To check the stability of the local chemotactic gradient, we determined $\langle \sin\varphi \rangle$ and $\langle \cos\varphi \rangle$ as a function of time (Fig. 6). As already mentioned by Zigmond (2), the chemotactic gradient is not established before 10 min after starting the experiment (dashed line in Fig. 6). As expected, $\langle \sin\varphi \rangle$ and $\langle \cos\varphi \rangle$ scattered around zero. The local chemotactic gradient is stable during the next 20 min since $\langle \sin\varphi \rangle$ was within the range of statistical error. However, at 45 min in this experiment $\langle \sin\varphi \rangle$ showed greater variations indicating local changes of the chemotactic gradient. The time dependence of $\langle \sin\varphi \rangle$ exhibits a trend that indicates a developing disturbance of experimental conditions, although the degree of orientation remains stable or even increases.

Directed Movement

Up to now we discussed (*a*) the degree of orientation, P_1, which represents basically only a static information, and (*b*) the track velocity, V_c, which describes the speed of nondirected movement. According to Eq. 11, both values are connected and their product, $V_\parallel$, defines the directed movement (chemotaxis). Insertion of the different experimental values of V_c and P_1 into Eq. 11 yields the following results of $V_\parallel$:

P_1	× V_c	= $V_\parallel$,	
0.80 ×	30 μm/min	= 24.0 μm/min	37°C,
0.40 ×	5 μm/min	= 2.0 μm/min	22°C,
0.26 ×	30 μm/min	= 7.8 μm/min	ECHO 9 A. B. virus (result from Fig. 5 *b*).

These data demonstrate that V_c and P_1 exhibit different sensitivities with respect to the virus and temperature. From these findings we conclude that the data processing for V_c is at least partly different from those of P_1. This means that a change in the chemotactic response of PMN described by $V_\parallel$ can be due either to changes of the track velocity V_c (as in the case of 22°C) or to changes of P_1 (as in the case of 22°C or ECHO 9 A. B. virus). That means that for better control of experimental conditions and to avoid misinterpretations of in vitro results, not only the degree of orientation, P_1 but also V_c and/or $V_\parallel$ should be determined.

Virus and Directed Movement

The chemotactic response of moving granulocytes is disturbed by ECHO 9 A. B. virus. We found that the track velocity of virus-treated PMN is not lowered; however, the degree of orientation declined. Debets-Ossenkopp et al. (19) used a different in vitro assay (under-agarose technique) for studying the interaction of influenza A virus with human PMN. They also found a decreased chemotactic response of virus-treated granulocytes. It is possible to interpret their results with our mathematical model: Under the likely assumption that V_c is constant in their experiments, the degree of orientation is decreased by a factor of 0.42 indicating, too, a shift from chemotactic to chemokinetic cellular response.

We demonstrated that the degree of orientation declines with increasing virus dose under otherwise constant experimental conditions. This result underlines the pathogenetic importance of the virus-induced change in cell behavior. A further observation confirms the pathogenetic role of virus: At a given dose the degree of orientation declines with the increase in duration of virus-granulocyte interaction. The degree of orientation decays with time exponentially. It was possible to define a critical virus/granulocyte ratio of 0.8 ± 0.1 pfu/PMN, indicating that there are two different states of virus-granulocyte interaction. At a virus dose below this critical value there is no disturbance of chemotactic response. But we do not know which other cellular events are influenced. Above this critical dose there is a virus dose-dependent decrease of the degree of orientation where the mean velocity is not affected. So far, the virus-induced disturbance of chemotactic response is described by Eqs. 11, 17, and 18 leading to a better understanding of virus-PMN interaction.

All our experimental data of the granulocyte-virus interaction with regard to the disturbed chemotactic cellular response are summarized in Eq. 18. This equation describes the dependence of the characteristic time, τ, on the viral dose, a. Rewriting Eq. 18, we get an equation that has the same structure as the Weber-Fechner law (27, 28):

$$\ln a - \ln a_0 = \frac{\tau_0^2}{\tau^2}. \quad (23)$$

The Weber-Fechner law connects the stimulus difference with the perceptive intensities. In our case, $\ln a_0$ defines the threshold value of the virus stimulus for the PMN. Consequently, $\ln a$ describes the virus stimulus for the granulocyte. The difference between any virus stimulus and the threshold value of virus stimulus is yielded by the square of the ratio τ_0/τ. τ describes the characteristic virus-induced decay time of the degree of orientation for a fixed viral dose, a. Analogous with the Weber-Fechner law, $(1/\tau)^2$ can be used as the perceptive intensity characterizing the virus-PMN interaction. However, τ_0 is not the virus-induced characteristic decay time of the threshold viral dose, a_0, because in this case the characteristic time is infinite. At this point, there is a deviation from the Weber-Fechner law. However, the characteristic time, τ_0, occurs at a fixed viral dose that is e-times the viral threshold dose, a_0 ($a_0 \times 2.718\ldots$). In the Weber-Fechner law the perceptive intensity is normally derived from subjective information. In contrast, the virus perception of PMN is derived from objective experimental data obtained from the virus dose- and time-dependent changes of the degree of orientation. So far, the virus stimulus, $\ln a$, and the perceptive intensity, $(1/\tau)^2$, are based on objective measurements.

Aside from the general interest of our experimental findings about the interaction of viruses with moving cells, some practical clinical aspects have to be discussed: Several studies have indicated that there is a transient depression of leukocyte locomotion during the course of a viral infection (20). Several in vitro investigations with a variety of viruses confirm these in vivo results (7, 19, 20). Experience in the daily work in human and experimental pathology tells us that viral infections are histologically characterized by a nonpurulent (i.e., weak or absent granulocytic) inflammation (21, 22). Many viral infections, e.g., influenza virus, predispose to bacterial superinfections (19, 23). It has been suggested that the increased susceptibility to bacteria is due to the effect of viruses on the immune system and on phagocytic cells (24–26). The demonstrated disturbances of granulocyte function in our investigations support this theory. The selective inhibition of chemotactic reactivity of virus-treated PMN provides a basis for the understanding of the morphological and clinical findings in viral disease.

Conclusion

By means of our experimental assay and using our statistical model, the McCutcheon index and the chemotropism index can be placed in a better perspective. Besides the analysis of static values of the experimental assay of moving PMN, more information about dynamic properties of the in vitro system is available, leading to a better description of experimental conditions. Consequently, application of this model to clinical investigations e.g., ECHO 9 A. B. virus interaction with human granulocytes results in a more vigorous description and interpretation of experimental findings.

We are indebted to Miss W. Riedel, Mr. H. Maute, and Mr. F. Gnann for excellent technical assistance.

Received for publication 25 June 1982, and in revised form 19 January 1983.

REFERENCES

1. Wilkinson, P. C. 1974. Chemotaxis and Inflammation. J. & A. Churchill Ltd., London. 3–22.
2. Zigmond, S. H. 1977. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J. Cell Biol.* 75:606–616.
3. Alt, W. 1980. Biased random walk models for chemotaxis and related diffusion approximation. Math. Biosci. 9:147–177.
4. Dahlquist, I. W., P. Lovely, and D. E. Koshland Jr. 1972. Quantitative analysis of bacterial migration in chemotaxis. *Nat. New Biol.* 236:120–123.
5. Keller, E. F., and L. A. Segel. 1971. Traveling bands of chemotactic bacteria: a theoretical analysis. *J. Theor. Biol.* 30:235–248.

6. Zigmond, S. H., and J. G. Hirsch. 1973. Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. *J. Exp. Med.* 137:387–410.
7. Bültmann, B., H. J. Eggers, and O. Haferkamp. 1981. Selective inhibition of human neutrophil chemotaxis by Echo-virus, type 9. *Klin. Wochenschr.* 59:571–573.
8. Zigmond, S. H. 1978. Chemotaxis by polymorphonuclear leukocytes. *J. Cell Biol.* 77:269–287.
9. Valone, F. H. 1980. Modulation of human neutrophil and eosinophil polymorphonuclear leucocyte chemotaxis. An analytical review. *Clin. Immunol. Immunpathol.* 15:52–65.
10. Eggers, H. J., and A. B. Sabin. 1959. Factors determining pathogenicity of variants of ECHO 9 virus for newborn mice. *J. Exp. Med.* 110:951–967.
11. Eggers, H. J., B. Bode, and D. Brown. 1979. Cytoplasmic localization of the uncoating of picornaviruses. *Virology.* 92:211–218.
12. Nahas, G. G., M. L. Tannieres, and J. F. Lennon. 1971. Direct measurement of leucocyte mobility: effect of pH and temperature. *Proc. Soc. Exp. Biol. Med.* 138:350–352.
13. Marasco, W. A., E. L. Becker, and J. M. Oliver. 1980. The ionic base of chemotaxis. Separate cation requirements for neutrophil orientation and locomotion in a gradient of chemotactic peptide. *Am. J. Pathol.* 98:749–768.
14. Haar, D. ter. 1955. Foundations of statistical mechanics. *Rev. Mod. Phys.* 27:289–338.
15. McCutcheon, M. 1946. Chemotaxis in leucocytes. *Physiol. Rev.* 26:319–336.
16. Nossal, R., and S. H. Zigmond. 1976. Chemotropism indices for polymorphonuclear leukocytes. *Biophys. J.* 16:1171–1182.
17. Ramsey, W. S. 1972. Analysis of individual leucocyte behaviour during chemotaxis. *Exp. Cell Res.* 70:129–139.
18. Zigmond, S. H. 1974. Mechanisms of sensing chemical gradients by polymorphonuclear leucocytes. *Nature (Lond.).* 249:450–452.
19. Debets-Ossenkopp, Y., W. C. van Dijk, E. L. Mills, H. A. Verbrugh, and J. Verhof, 1980. The effect of influenza virus on human polymorphonuclear leucocytes. *Antonie Leeuwenhoek J. Microbiol.* 46:103*a*.
20. Wilkinson, P. C. 1980. Leucocyte locomotion and chemotaxis: effects of bacteria and viruses. *Rev. Infect. Dis.* 2:293–318.
21. Bültmann, B., J. Galle, H.J. Eggers, and O. Haferkamp. 1981. Ultrastructural degenerative changes of age dependent Echo 9 virus-induced polymyositis in infant mice. *Virchows Arch. B. Cell Pathol.* 35:177–187.
22. Budding, G. J. 1969. Pathogenesis and pathology of viral infections. *In* Viral and Rickettsial Infections of Man. F. L. Horsfall and J. P. Lippincott, Philadelphia. 339–356
23. Martin, C. M., C. M. Kunin, C. S. Gottlieb, and M. Finland. 1959. Asian influenza A in Boston, 1957–1968. II. Severe staphylococcal pneumonia complicating influenza. *Arch. Intern. Med.* 103:532–542.
24. Larson, H. E., and R. Blades. 1976. Impairment of human polymorphonuclear leucocyte function by influenza virus. *Lancet.* 1:283.
25. Stuart-Harris, C. H. 1979. The influenza viruses and the human respiratory tract. *Rev. Infect. Dis.* 1:592–599.
26. Anderson, R., R. Sher, and H. J. Koornhof. 1976. Neutrophil motility in children with measles. *J. Pediatr.* 89:27–32.
27. Gauer, O. H., K. Kramer, and R. Jung, editors. 1974. Allgemeine Neurophysiologie. Urban und Schwarzenberg Verlag, Munich. 10:193.
28. Carterette, E. C., and M. P. Friedman, editors. 1974. Handbook of Perception. Academic Press, New York. 2:197.