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NEUTROPHIL LEUCOCYTE CHEMOTAXIS IS NOT INDUCED BY A SPATIAL GRADIENT OF CHEMOATTRACTANT

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SUMMARY

Chemotaxis and directed locomotion of neutrophil leucocytes are generally thought to be determined by the directed response of the cell to stable, spatial gradients of chemoattractants. In most cases, however, cells are also exposed to characteristic temporal changes in the attractant concentration during the lifetime of the gradient, especially as it develops. We have attempted to test whether neutrophils can respond to a spatial gradient in which these temporal changes are essentially absent. Gradients of formyl-peptides were made across a narrow barrier of agarose gel that separated two fluid reservoirs, and the cells were observed cinematographically as they moved between gel and glass. In gradients predeveloped at low temperature, at which cell motion and responses to attractant were inhibited, neutrophils showed no tendency to accumulate up-gradient when warmed to 37 °C. Yet their speed and turning behaviour was related to the local concentration of formyl-peptide. However, gradients that developed at 37 °C, whilst the cells were responsive, elicited directed locomotion. We also tested populations that were either spreading into or already evenly distributed across micropore filters to see how cells might sense directional cues. We reasoned that evenly distributed populations could accumulate in a spatial gradient only if cells were able to 'read' it. However, no redistribution occurred without an applied impulse of attractant. It seems that the oriented, temporal component of an attractant signal is essential if a directed response (i.e. non-random turning) is to occur; a spatial gradient of soluble attractant alone does not induce neutrophil accumulation or taxis. This finding has implications for the termination of the acute inflammatory response, for clinical tests of leucocyte behaviour and for morphogen signal interpretation by cells in developing tissues.

INTRODUCTION

All but the most primitive metazoa require migratory phagocytes in order to resist infection. These motile cells accumulate at sites of inflammation, often demonstrating a remarkable degree of directional locomotion (for reviews, see McCutcheon, 1946; Zigmond, 1978; Wilkinson, 1982). The nature of cellular orientation, attraction and accumulation responses has interested many workers including Leber (1888), who, referring to his observations on neutrophils, was perhaps the first to express the view that "chemotaxis (or) the direction of movement . . . is influenced by the concentration difference" of the attractant. The

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term 'chemotactic gradient' has, subsequently, been invoked as an explanation for any attractant-induced orientation or accumulation of cells. Thus, partly because of this conflation, both the signal form and the mechanism of signal perception are usually assumed to be self-evident. This state of affairs has developed despite the efforts of a few authors who have pointed out that leucocyte emigration from vessels and accumulation at sites of tissue damage are not necessarily due to chemotaxis (Harris, 1954; Keller & Sorkin, 1968; Wilkinson *et al.* 1982).

The concept of spatial gradients of diffusible molecules as signals has been highly influential and is often discussed as the basis for the specific cell migration, positioning and differentiation that characterizes many morphogenetic processes (Wolpert, 1969; Crick, 1970; Meinhardt & Gierer, 1980). Only a few cell types, however, have been shown to react unambiguously to attractant or morphogen fields. Among these are some motile bacteria (McNab & Koshland, 1972; Berg & Brown, 1974) and the classical eukaryotic models of chemotactic behaviour: the neutrophil leucocytes (Wilkinson, 1982) and the cellular slime moulds, in particular *Dictyostelium discoideum* (Gerisch *et al.* 1975).

Accumulation in bacteria is driven by the anisotropic stimulation of their adaptive motor reactions induced in spatial gradients of attractant (Berg & Brown, 1974; Koshland, 1977). As bacteria swim into or encounter higher concentrations they tend to swim more persistently in straighter tracks, i.e. they turn (tumble or 'twiddle') less frequently; but persistence deteriorates as they become exposed to lower or constant concentrations. This behaviour apparently results from rapid adaptation to the concentration, which suppresses the motor reactions responsible for persistence (Berg & Tedesco, 1975). Accumulation occurs because the cells propel themselves into the higher concentrations of attractant at a rate exceeding the relaxation rate of the adaptive processes and, thus, fast enough to inhibit turning. The response is, therefore, klinokinetic–adaptive behaviour based on perception of this self-generated temporal gradient. Bacteria are not tactic, since their direction after each turn is assumed randomly.

A different mechanism of gradient perception was originally supposed to control taxis in crawling cells, such as *D. discoideum* (Bonner, 1947) and neutrophils (Zigmond, 1974; and see Zigmond, 1977), in which directed turns were made in response to the concentration difference of chemotactic factor across the length of the cell. This 'spatial' mechanism has recently fallen out of favour, and a 'temporal' mechanism, analogous to that postulated for bacteria, has since been proposed for both *D. discoideum* (Gerisch *et al.* 1975) and neutrophils (Gerisch & Keller, 1981; Dunn, 1981; Zigmond *et al.* 1982). The suggestion is that a rapidly advancing pseudopod probes the environment much like an advancing bacterium: as the tip of the pseudopod moves up-gradient the sharp rate of increase in the attractant concentration increases persistence by stimulating reactions that suppress the effects of adaptation. In decreasing or constant concentrations, e.g. if the pseudopod is directed down-gradient, the reactions remain at or return to equilibrium.

The hypotheses of chemotactic-gradient perception rest on particular suppositions concerning the nature of the spatial gradient signal, e.g. its identification with a

stable, linear, diffusion gradient. However, in most or all studies motile cells have been confronted with a developing gradient (i.e. an attractant impulse or, rather, a temporal gradient) during the experiment. Furthermore, the propagation of signals in low-viscosity media is only partially governed by the law of diffusion; more significant for the movement of attractant are perturbations due to convection, turbulence and the pooling and flow of attractant (Vicker, 1981).

In an attempt to overcome these difficulties Vicker *et al.* (1984) examined the behaviour of *D. discoideum* under conditions in which cells were exposed either to developing attractant gradients or to predeveloped, spatial gradients alone. Accumulation required an impulse of chemoattractant (cyclic AMP), but did not occur in populations exposed to steep, predeveloped spatial gradients. These results prompted us to examine the behaviour of neutrophil leucocytes. In this paper we report experiments in which we exposed neutrophil leucocytes to different attractant signals: (1) to gradients developing at 37°C (an impulse) in which the concentration is rapidly changing near the source of attractant, and which we designate temporal gradient signals; or (2) to gradients predeveloped at low temperature and essentially stabilized before the cells are exposed to them at 37°C. These gradients show only small changes over long time periods and we designate them spatial-gradient signals. We have sought to discover which specific form of attractant signal can induce a particular type of cell response.

MATERIALS AND METHODS

Cells and media

Leucocytes (95% polymorphonuclear neutrophils (PMN)) were harvested from the peritoneum of New Zealand White rabbits 4 h after the injection of 400 ml of 1% glycogen (Sigma, UK) in 0·9% saline (Lackie, 1974). Cells were stored in the peritoneal exudate (PEX) at 2°C and used within 3 days. Media consisted of either (1) EPEX: Eagle's medium buffered with 20 mM-Hepes (Gibco, Paisley, Scotland) plus an equal volume of PEX; or (2) HPEX: Hanks' saline rather than Eagle's. Agarose (1%, 450 g cm^{-2}, low sulphate; Marine Colloids, Stokes Poges, England) was prepared in HPEX and set in glass or tissue culture grade plastic Petri dishes. The attractant formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) was from Sigma (UK).

Micropore filter migration chambers and gradient generation

Filter circles (3 μm pore diameter: Schleicher & Schüll, UK) were cut with a paper-hole punch and glued with Uhu to the cut ends of 1 ml plastic tuberculin syringe barrels from which the markings had been removed (see Appendix, Wilkinson, 1982). About 2×10^5 cells in 0·2 ml HPEX, with or without fMet-Leu-Phe, were added to this 'chamber' and 2·5 ml to the lower chamber (a 5 ml glass beaker) on ice (Fig. 1A and Wilkinson *et al.* 1982). The filter chamber was placed in the beaker at equal liquid levels and, after 10 min on ice to enable cells to settle while restraining their motility, they were warmed to 37°C. Thus, although the gradient is propagated with its developing temporal and spatial components, the cells treated by this technique should not sense the sharp initial temporal gradient.

In order to make sure that they were in a state necessary to sense a developing gradient, both the upper and lower chambers were warmed to 37°C before combination so as to expose cells to developing gradients while they were motile. We term the signals generated by the method above spatial gradients and those here temporal gradients for operational reasons, since the spatial gradient is present at 37°C in both cases. Indeed, even in 'stable' spatial gradients some degradation or perturbation occurs; however, we believe these insignificant compared to the initial temporal

gradient at our level of observation. After 30–60 min incubation, the filter chambers were drained and immersed in 70% ethanol for cell fixation and to dissolve the glue. The filters were washed twice in water, stained for 5 min in Giemsa, washed three times in water, dried and mounted with Permount (Fisher; Fairlawn, New Jersey, USA). Cell distributions within filters were scored with a 40×, 0·65 n.a. objective and Köhler illumination, beginning 4–25 μm beneath the filter surface and proceeding in optical sections of 15–25 μm (Zigmond & Hirsch, 1973; Vicker *et al.* 1984).

Evenly distributed neutrophil populations were produced in vertical micropore filters in order to avoid losses of cells from the lower surface. A 15 mm diameter filter (5 μm pore size) and neoprene gasket were sandwiched between two blocks of Perspex (Lucite) and, thus, separated two 0·7-ml chambers (Fig. 1B). The whole filter block was positioned horizontally while a 0·5 ml neutrophil suspension in PEX (10^6 cells ml^{-1}, passed first through a 10-μm nylon mesh: Nitex, Plastok Associates, Birkenhead) was added to the uppermost chamber at 21°C. Gentle suction from the lower chamber drew the cells onto the filter surface. After 3 min the block was inverted, a cell suspension was added to the upper chamber and the operation was repeated. The filter block was then placed vertically with 0·7 ml EPEX and 10^{-11} M-fMet-Leu-Phe in both chambers. Cells were incubated at 37°C for 3 h, until they had evenly permeated the filter. The block was then cooled on ice 10 min before the addition of 2×10^{-9} M-fMet-Leu-Phe in 70 μl EPEX to one or both chambers. Following gradient development (after 5 min), incubation was continued in a 37°C room for 30–60 min. To expose motile cells to developing gradients, fMet-Leu-Phe was added to one chamber only after the cells had been warmed to 37°C. Incubation was then continued for 20–50 min, and cell distributions were measured as above. The total incubation time at 37°C for cells exposed to developing gradients is kept identical to that for those treated with predeveloped ones. Thus, the actual time of exposure to the developing gradient signal is necessarily less than

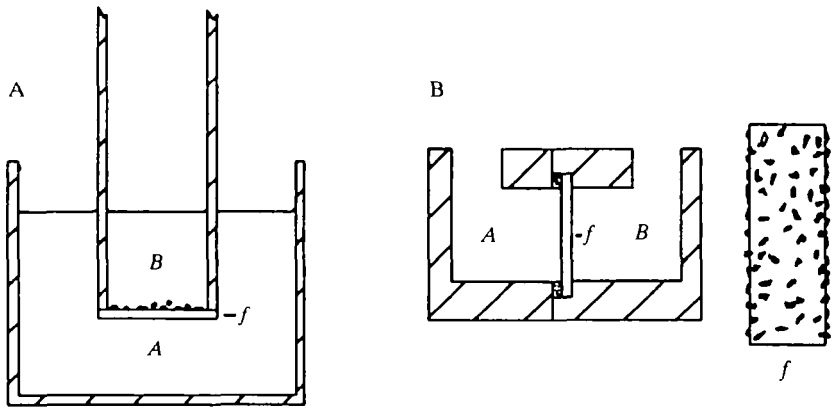

Fig. 1. Micropore filter territories for neutrophil locomotion. Two chamber types were constructed. A. Filter penetration by a spreading cell population. A cell suspension was added to the *B* chamber ice-cold and the cells were allowed to settle upon the 3 μm pore diameter filter (*f*). For isotropic gradients, the chambers each contained 2×10^{-9} M-fMet-Leu-Phe in HPEX. For predeveloped spatial gradients, 4×10^{-9} M-fMet-Leu-Phe was included only in chamber *A*. Chamber *B* was then placed in *A* (ice-cold) and, after incubation on ice for 10 min to permit complete gradient development, the assemblies were transferred to a room at 37°C. Temporal gradients were generated in motile populations by pre-warming chambers *A* and *B* (*B* was placed in a bath of HPEX) to 37°C before inserting *B* into *A*, which contained 4×10^{-9} M-fMet-Leu-Phe. B. Randomly distributed populations were developed in 5 μm pore diameter filters, which were incubated vertically between two chambers within a block of Perspex. The stippled area represents a neoprene gasket. An enlarged diagram of the territory is shown on the right. The block was cooled on ice before isotropic, predeveloped spatial or temporal gradients were generated across the filter as in A above.

that to the predeveloped one. The form, development and stability of gradients in micropore filters and in agarose gels have been discussed by Vicker (1981).

Time-lapse filming

A filming chamber consisted of a stainless steel slide 0·7 mm thick with a 15 mm diameter hole in the centre. A glass coverslip was affixed to the underside with silicone grease, and 0·2 ml cell suspension in HPEX (1×10^5 to 2×10^5 cells ml^{-1}) was added (Wilkinson *et al.* 1982). After cell attachment, a block of 1 % agarose (15 mm × 2 mm × 1 mm) in HPEX was gently placed upon the cells, forming a barrier across the diameter of the chamber. A few microlitres of molten agarose were used to seal the barrier to the coverslip, and the wells on each side were filled with HPEX, with or without fMet-Leu-Phe, on ice. A coverslip was placed upon the chamber, excess fluid was removed and the coverslip was sealed with molten paraffin wax/vaseline (60:40, w/w). The gradients were permitted to develop for 2 h at 2°C until virtually linear (Vicker, 1981).

Each chamber was warmed to 37°C for 15 min before filming. An air-curtain incubator was used to maintain a temperature of 37(±0·1)°C on the stage of a Leitz Ortholux microscope. A motorized 16 mm Bolex camera, controlled by an intervalometer, produced 0·2-s exposures at 6-s intervals, using Kodak Plus-X film. A 10× phase-contrast objective was used to film fields of low population density, which helped to keep cell collisions to a minimum.

Cell distribution and track analysis

The analysis of cell migration within micropore filters required a calculated one-dimensional distribution of cells in grouped form as before (Vicker *et al.* 1984), and 350–900 cells were counted in 5–12 fields/filter. Track analysis of the paths and displacements of cells on film was done essentially by the methods of Lackie & Burns (1983) and Wilkinson *et al.* (1984). For any one concentration of fMet-Leu-Phe and treatment, 15–25 cells were selected per field and their motion recorded by dotting in their tracks at 1-min intervals for 30 min each using an analytical projector (L & W Photo Optical Data Analyser, Van Nuys, CA, USA). The coordinates of the position of each cell after each 60 s were directly entered into a microcomputer using a digitizing tablet (Lackie & Burns, 1983). These were used to calculate the root-mean-square cell speed (S), directional persistence time (P) and the rate of diffusion (R), where $R = 2S^2P$ (Wilkinson *et al.* 1984).

RESULTS

Filter penetration by spreading populations

Neutrophils possess an impressive agility in being able to writhe their way through the tortuous labyrinth of a micropore filter, even one of 3 μm pore diameter or less. In current practice (Zigmond & Hirsch, 1973) cells are placed on the upper surface of the filter and the effect of an attractant on cell motility is assessed by the rate or degree of penetration. The nominal 150 μm thickness of such filters supports a linear, chemical gradient whose steepness, as $\Delta C/C$ across a cell, increases as one approaches the 'sink' chamber, and which is considerably steeper and more stable than a gradient attainable with virtually any other experimental design.

Isotropic concentrations of fMet-Leu-Phe slightly increase the mean penetration of neutrophils compared to HPE alone (Figs 2, 3). Cells may be detected migrating into the filter within a few minutes at 37°C, but the effect of fMet-Leu-Phe is greater after longer incubations. Maximal penetration results from exposing cells to a single impulse of fMet-Leu-Phe. However, when cells are cooled on ice during gradient generation, and are immotile during its development, their degree of penetration is

similar to that in an isotropic concentration. Presumably, cooled cells are either insensitive to the effects of the temporal gradient or their adaptive reactions have relaxed to equilibrium before warming to 37°C. The proportion of motile cells is

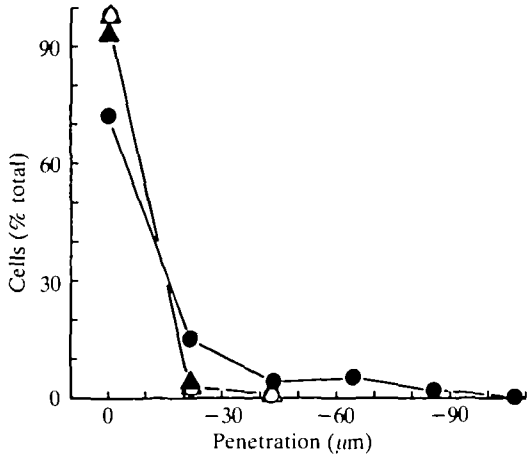

Fig. 2. Filter penetration by spreading neutrophil populations. The experiment was conducted as in Fig. 1A so that after being warmed to 37°C, populations (about 2×10^5 cells) were exposed to either HPEX alone (▲), an isotropic concentration of fMet-Leu-Phe (△) or a predeveloped spatial gradient of fMet-Leu-Phe (○). The latter gradient, as $\Delta C\,\mu m^{-1}$, was $\approx 2{\cdot}7\times10^{-11}$ M-fMet-Leu-Phe μm^{-1} or, across an extended cell, $\approx 4\times10^{-10}$ M per 15 μm. The gradient's steepness (as $\Delta C/C$) was 20 % across a cell at mid-filter. Migration continued for 40 min until fixation. Some prewarmed populations were exposed to a developing fMet-Leu-Phe gradient (●) for a total of 30 min after addition of fMet-Leu-Phe. The cell distributions were measured in optical sections at intervals through the depth of the filters; the upper surface being at 0 μm.

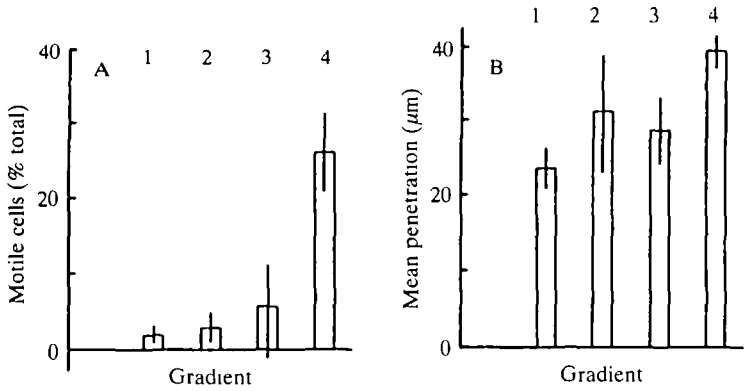

Fig. 3. Filter penetration by spreading populations: a summary. A. The number of migrating cells (as proportion of the population), and B, the mean depth of penetration are given with their standard deviation indicated by vertical bars (±S.D., $n = 4$). Experiments were conducted as in Figs 1 and 2 with cells exposed to various gradients: 1, HPEX alone; 2, an isotropic fMet-Leu-Phe concentration; 3, a predeveloped spatial gradient of fMet-Leu-Phe (one developed on ice); or 4, an impulse (a gradient developing at 37°C) of fMet-Leu-Phe.

more sensitive to the type of fMet-Leu-Phe signal (more apparent in short incubations). A single impulse stimulates nearly 30% of the population, but in populations exposed to a spatial gradient or to an isotropic concentration the proportion of stimulated cells is very little different from that of populations incubated in HPEX alone.

Shifts in randomly distributed populations

The results described above, using spreading populations, indicate that quantitatively different effects exist between the different types of fMet-Leu-Phe signals. In order to demonstrate that these differences are also qualitative, it is necessary to use randomly distributed cell populations confined in bounded territories. Inability to leave the territory will prevent spreading of the population. If cells are able to read the orientation of a signal, e.g. a spatial gradient applied across such a territory, they ought to accumulate. The ability of cells to break the equilibrium of a random distribution should, thus, distinguish unequivocally between their various possible signal-reading capacities.

The results of these experiments are presented in Figs 4 and 5. When evenly distributed populations are exposed to a predeveloped spatial gradient of fMet-Leu-Phe there is no change in either the mean or median cell position. Only a developing gradient (an impulse) perturbs the cell distribution and induces a population shift up-gradient. The shift of the median is highly significant. Therefore, in regard to directional cues, evenly distributed neutrophil populations appear unable to interpret soluble spatial gradients and are only sensitive to the temporal gradient delivered by a directed impulse of fMet-Leu-Phe, which induces attraction and directed locomotion.

Cell speed and persistence in spatial gradients of fMet-Leu-Phe

A film analysis of individual cell tracks was necessary both in order to test the results of the filter experiments and to record the parameters of locomotion under different signals. Previous film observations have demonstrated that neutrophils home accurately toward emitters of chemoattractants. Such emitters usually confront cells with developing and, or, chaotic gradients. Therefore, we sought to repeat these experiments on populations exposed only to a spatial gradient signal; one in which the effects of the temporal gradient on cell reactions had been suppressed by predevelopment and instabilities in the gradient eliminated by propagation in an agarose gel.

Under such conditions fMet-Leu-Phe increases cell speed (S), persistence (P) and diffusion (R) of the population as the concentration approaches the optimum of about 2×10^{-9} M (Fig. 6). Thus, fMet-Leu-Phe induces both positive orthokinesis and negative klinokinesis. The gradient (as $\Delta C/C$, where ΔC is the concentration difference across a cell) is equally steep at each concentration tested. If agarose is not used, and cells are not constrained dorsally, the values of all the parameters are decreased by about half. There is no correlation between the enhancement of locomotion (S, P or R) and the amount of cell flattening under agarose, as measured

by the average plane cell area of 20–30 cells at each of the five concentrations of fMet-Leu-Phe tested (data not shown). Movement under agarose probably inhibits all but lateral pseudopodial projections and seems to eliminate the elastic 'snap-back' of cells fastened by tenacious retraction fibres. This benefits the analysis, but we can only conjecture about its cause.

Cell directionality in spatial gradients of fMet-Leu-Phe

Attraction and accumulation in response to the 'reading' of a spatial gradient would cause a net population displacement and an increase in directed movement by the individual cell. The population displacement was estimated by summing the vectors of displacement for all the cells, the displacement being the difference between the starting and finishing positions of the cell (in the film). No difference in net population displacement was found between cells exposed to either spatial gradients or matching isotropic concentrations (Fig. 7). The average directionality of all the

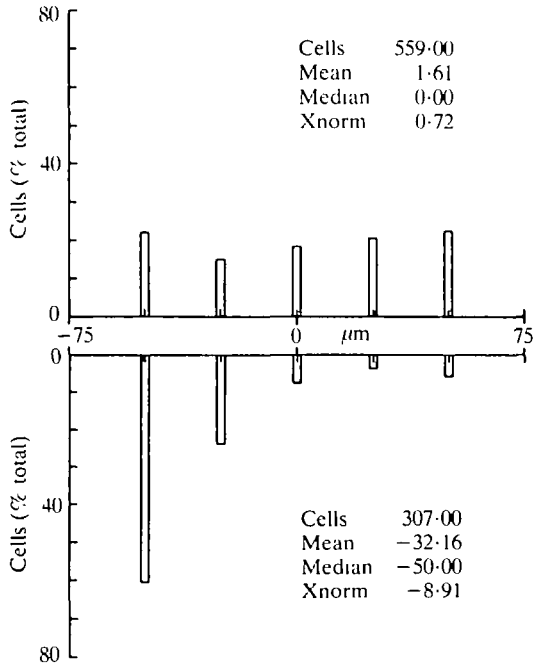

Fig. 4. Comparison between the effects of a predeveloped spatial fMet-Leu-Phe gradient and an fMet-Leu-Phe impulse on the cell distribution of initially evenly distributed (equilibrium) populations. Two filter territories were permeated with cells as in Fig. 1B and then exposed either to a predeveloped spatial gradient for 38 min (upper panel) or to a gradient developing at 37 °C for 23 min (lower panel). fMet-Leu-Phe was added to the left side of the filter at −75 μm. Each panel gives (1) the number of cells counted in the filter (cells on either filter surface at −75 μm and +75 μm are not included); (2) the resultant mean position of the population (in μm); (3) the resultant median position (in μm); and (4) the significance and direction of the median shift by the Wilcoxon signed rank test (Xnorm). Values greater than |2·56| are significant at the 1 % level.

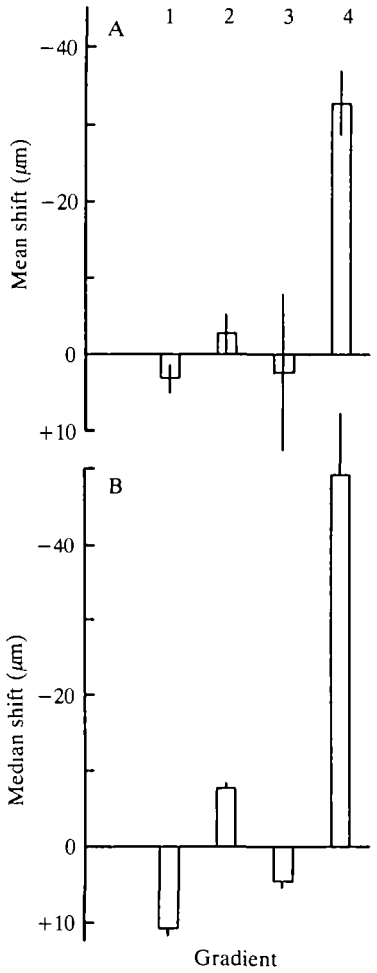

Fig. 5. A summary of the effects of fMet-Leu-Phe signals on initially evenly distributed (equilibrium) neutrophil populations. A. The mean positions of several populations are averaged and given with the S.D. Populations were exposed to various gradients: 1, HPEX alone (range only, $n = 2$); 2, an isotropic concentration of fMet-Leu-Phe ($n = 5$); 3, a predeveloped spatial gradient ($n = 6$); or 4, an impulse ($n = 3$). B. The median position is given with the direction and value of Xnorm. Treatments correspond with the lane numbers in A. The experiments were conducted as in Figs 1B and 4.

cells in each film sequence indicates little to differentiate it from random locomotion. We obtained the same results by recording the cell direction after each 1-min 'step' (data not shown). Directed locomotion and accumulation were inducible in neutrophils in these film chambers by injecting fMet-Leu-Phe locally through a narrow (1 μm internal diameter) capillary micropipette (our unpublished observations; and Gerisch & Keller, 1981). Thus, spatial gradient signals are interpreted by cells as isotropic concentrations: the reaction is biased-random locomotion (kinesis) in both cases. Chemotaxis apparently requires an impulse or pulse signal: a temporal gradient.

DISCUSSION

In this paper we have demonstrated the orthokinetic and klinokinetic effects of fMet-Leu-Phe on neutrophil leucocytes, and then shown that these cells seem incapable of responding by taxis to spatial gradients of this supposedly chemotactic factor. The results on the stimulation and alteration of random movement are in accord with those of other authors (McCutcheon, 1946; Ramsey, 1972; Zigmond &

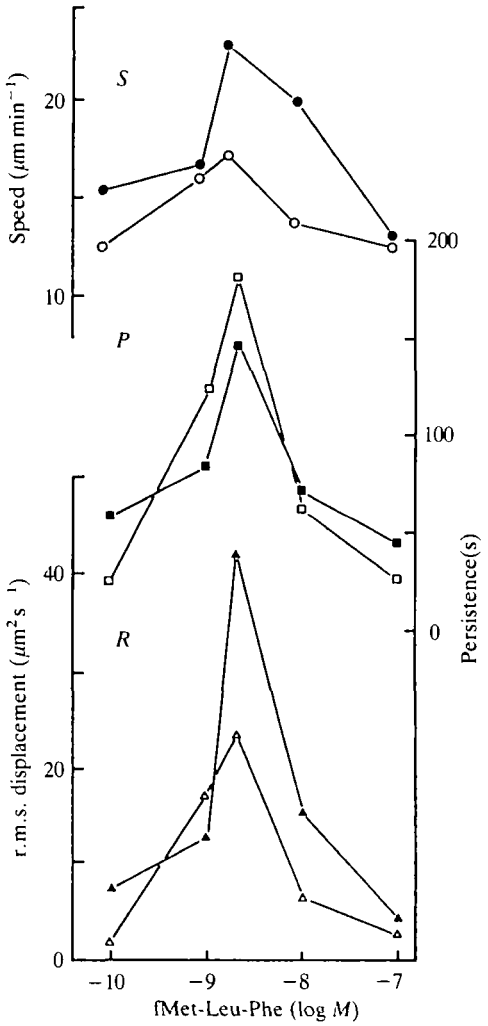

Fig. 6. Locomotion parameters of neutrophils migrating in predeveloped spatial gradients of fMet-Leu-Phe under agarose. The solution of fMet-Leu-Phe in HPEX was added to one side of the agarose barrier and HPEX to the other side. Therefore, the gradient, as $\Delta C\,\mu m^{-1}$, is $C_{max}/2000\,\mu m$. As $\Delta C/C$, the gradient's steepness is equal between the different concentrations and is $\approx 1{\cdot}5\,\%$ at mid-gradient. The average speed (S), persistence (P) and root-mean-square (r.m.s.) displacement (R), where $R = 2S^2P$, were calculated for 15–25 cells filmed at each concentration of fMet-Leu-Phe. The results are given from two experiments (open and filled symbols).

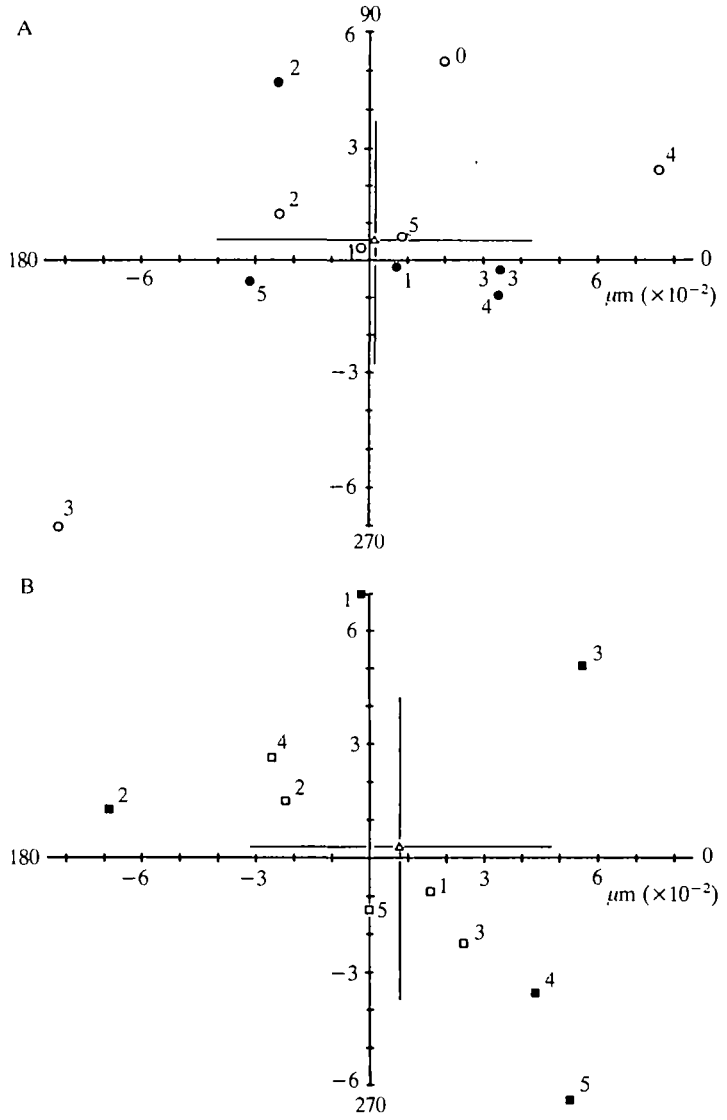

Fig. 7. Scattergrams comparing the first and last positions of neutrophils in isotropic concentrations (A) or predeveloped spatial gradients (B) of fMet-Leu-Phe under agarose. Each point represents the sum displacement of 15–25 cells measured at each concentration (from Fig. 6). For A, the molar concentrations of fMet-Leu-Phe were 0, 0 (HPEX alone); 1, 10^{-10}; 2, 10^{-9}; 3, 2×10^{-9}; 4, 10^{-8}; and 5, 10^{-7}. The concentrations of fMet-Leu-Phe for B are correspondingly greater by a factor of 2 (the mid-gradient concentration is, therefore, always equal to that of the matching isotropic concentration). fMet-Leu-Phe was added from a position corresponding to 90° on the scattergram. The results are shown from two experiments (open and filled symbols). The average sum displacement of all cells at all concentrations in A and B is indicated by an open triangle together with the S.D. for the X and Y directions, respectively.

Hirsch, 1973; Zigmond, 1974; Keller *et al.* 1977; Howard & Meyer, 1984), and are included to show that we could detect a response, that the parameters of motility depend on the altered concentrations of fMet-Leu-Phe, and that fMet-Leu-Phe was, therefore, present in the fields we observed. We used an under-agarose system and a microfilter-penetration system to stabilize developing and predeveloped fMet-Leu-Phe gradients. In the agarose system we found concentration-dependent effects of fMet-Leu-Phe on the speed and turning frequency ($1/P$) of neutrophils, and that at the gradient mid-point these parameters were similar to those for cells in a similar but isotropic concentration. However, when we recorded the paths of individual cells we could detect no up-gradient bias in their movement in predeveloped spatial gradients, nor was there any net population displacement.

Analysis of the behaviour of neutrophil populations in the micropore filter system shows that isotropic concentrations and spatial gradients of fMet-Leu-Phe are virtually equal in their ability to increase both the proportion of motile cells and the migration of cell populations spreading into micropore filters. However, a brief impulse of attractant is more effective. The essential qualitative difference between temporal and spatial signals is demonstrated by the inability of predeveloped, spatial gradients to perturb the distribution of evenly spread cell populations in bounded territories, while but one brief impulse is a powerful attractant. This shift is due neither to orthokinetic nor to klinokinetic effects (Vicker *et al.* 1984). Thus, although fMet-Leu-Phe affects the kinetic behaviour of neutrophils, a spatial gradient does not seem to elicit responses of the type predicted by the chemotactic-gradient hypothesis; namely, effecting the directed turning, orientation, attraction or accumulation of individual cells.

This surprising result apparently conflicts with much evidence that has been interpreted as demonstrating that fMet-Leu-Phe is a chemotactic factor and that neutrophils will respond to spatial gradients. However, the contradiction may be resolved if it is realized that under most experimental (and *in vivo*) conditions the attractant signal is commonly taken to be identical to the spatial gradient form that we tested, and temporal gradient effects are ignored. In fact, in most cases the gradient develops rapidly as the experimental observations proceed. Furthermore, in low-viscosity media it would be mistaken to attempt to predict its development or form, i.e. to assume that a stable spatial gradient even exists.

Comparison with the present concepts of gradient reading

As appealing as the previously suggested mechanisms of spatial gradient perception are (e.g. see Bonner, 1947; Gerisch & Keller, 1981), there remain acute problems with them, not least biochemically. With regard to the spatial mechanism, the observations of Keller & Bessis (1975), that even fragments of leucocytes home toward sources of attractant, indicate that there might be no lower limit to the cell length necessary to detect a spatial gradient. However, theoretical analysis of cell-surface receptor kinetics has shown that cells, including bacteria, should be able to detect even a rather shallow and 'noisy' spatial gradient by temporal integration of the cell surface receptor signals (Berg & Purcell, 1977). Lauffenburger (1982) came to

similar conclusions, but added that such integration should only occur in moving cells. With regard to the temporal mechanism, two points are relevant. (1) The hypothesis supposes that pseudopodia probe the environment at random. Consequently, turning would be random, and the result would be a klinokinetic–adaptive mechanism as in bacteria, not taxis. (2) The temporal mechanism does not require translocation of the whole cell, but rather that a portion of it be used as a probe, i.e. 'pseudo-spatial sensing' (Lackie, 1986). The observations of Zigmond (1974), that the first displacement of a neutrophil is usually up-gradient, appears the strongest evidence for a spatial gradient sensing mechanism, and is supported by the results of Ramsey (1972), Gerisch *et al.* (1975) and Gerisch & Keller (1981), which show that attractant delivered by micropipette near a cell immediately elicits the extension of a pseudopod toward the pipette, i.e. the cells are tactic. Yet, these four investigations only indicate that the whole cell might sense the direction of the gradient, not that sensing occcurs during pseudopod extension. This follows because pseudopods were selectively elicited and correctly oriented from the outset, and not selected from among those aimed towards and away from the signal source.

The results reported here are the first indication: (1) that chemotaxis in neutrophil leucocytes is not due to the spatial gradient of a chemoattractant; and (2) that temporal gradients play a crucial role. These aspects of neutrophil behaviour are manifest only through the application of defined spatial and temporal chemoattractant signals. Taxis appears specifically as the directed turning of individual cells in response to the direction of the temporal gradient (pulse or impulse) signal (Nossal, 1976; Nossal & Zigmond, 1976). We postulate that cells sense the directionality of a pulse or impulse signal, because it (a 'threshold' concentration) first contacts them on one side, and that before the signal reaches the other side sufficient time has elapsed (about 1 s would appear reasonable) to generate the cellular reactions necessary for cell polarization or directed pseudopodial extension (Vicker *et al.* 1984). However, the stochastic behaviour of neutrophils in spatial gradients of fMet-Leu-Phe, i.e. their biased-random walk and the wide individual variation in the degree of kinesis, indicates that the behaviour of individual cells there is governed by the local fMet-Leu-Phe concentration and not by the concentration gradient.

Two reasons make it appear unlikely that the directed response to developing gradients (impulses) is in fact due to nothing other than the reading of a very steep spatial gradient produced as the first, ephemeral 'wave' of attractant passes across the cell. (1) The relative steepness of a developing gradient across a cell (considering steepness as $\Delta C/C$, where ΔC is the concentration difference across the cell) is a constant, K, at any point in the gradient: picture an exponentially decreasing curve. Thus, it is not especially steep at any one point or at any one time during its early development; no wave or conspicuous, steep front actually exists. (2) In a linear gradient, such as that used in this study, relative steepness (as $\Delta C/C$) is not a constant, but increases continuously towards C_{min}: for example, from about 20% across a 15 μm-long cell extended at mid-filter to 50% at 120 μm from C_{max}. Thus, cells near the 'sink' chamber move in gradients approaching 100% (Vicker *et al.* 1984). The absolute steepness of a developing gradient (as ΔC), especially in its up-

gradient range, exceeds that of a linear one. Yet, developing gradients are at that point not steeper (as $\Delta C/C$) than the down-gradient range of a linear gradient. Furthermore, the attractant concentrations may be made to be comparable and optimal at the steepest (as $\Delta C/C$ or ΔC) range of either developing or static (linear) gradients; however, the latter induce neither cell accumulation nor taxis.

Adaptive reactions in neutrophils

The locomotory reactions of neutrophils to the different attractant signals are virtually identical to those of *D. discoideum* (Vicker *et al.* 1984). Neutrophils, like the slime mould, also express a sequence of adaptive reactions in specific response only to temporal signals of stimulant, including the binding of attractant receptor molecules to the cytoskeleton (Jesaitis *et al.* 1984), cytoskeletal changes and actin polymerization (Fechheimer & Zigmond, 1983; Sheterline *et al.* 1984; Wallace *et al.* 1984; Howard & Meyer, 1984; Howard & Oresajo, 1985), contraction (Zigmond & Sullivan, 1979; Shields & Haston, 1985), volume and adhesive changes (O'Flaherty *et al.* 1977; Forrester & Lackie, 1984), chemiluminescence (Descamps-Latscha *et al.* 1982) and protein phosphorylation (Slonczewski *et al.* 1985). We suspect that in spatial gradients of attractant the rate of increase in cell-surface receptor occupancy is too slow, even if a pseudopod is moving directly up-gradient, to stimulate these reactions and, thus, cannot drive accumulation like that observed in bacteria (which are about 100 times faster than neutrophils) or as predicted by the temporal mechanism hypothesis. This conclusion is supported by the observations of Zigmond & Sullivan (1979) that neutrophils contract upon contact with a developing fMet-Leu-Phe gradient, but not again as they continue their up-gradient migration. Significantly, the kinetics and specific temporal-signal sensitivity of taxis appear to match that of actin polymerization in neutrophils (Wallace *et al.* 1984). Such an immediate relationship might indicate: (1) a specific pathway for temporal signals between reception and G–F actin transition, which is absent in spatial gradient signals; and (2) a role for an endogenous oscillator, comprising elements of the cytoskeleton (Alt, 1985), in signal interpretation and cell response in neutrophils and amoebae like that regulating flagella reactions in the phototactic *Halobacterium* (Schimz & Hildebrand, 1985).

Other reactions to spatial gradients

An example of directed locomotion in a spatial gradient unequivocally devoid of temporal components has been reported by Wilkinson *et al.* (1984). Neutrophils were plated upon a surface-bound step-gradient composed of two areas, one of which was coated with immunoglobin and to which they were more adhesive (through their Fc receptors). The cells accumulated over that area where they were most adhesive and where they also moved slowest (S); however, the persistence time (P) did not vary as they changed areas, and turning direction was random. Analogous experiments with neutrophils were conducted by Dierich *et al.* (1977) and Wilkinson & Allan (1978). Similar behaviour in fibroblasts was termed 'haptotaxis' by Carter (1967), but Harris (1973) reported that fibroblasts moving upon an adhesive gradient

were often apparently randomly orientated despite their tendency to accumulate. Therefore, this form of reaction to a spatial gradient is not taxis, but rather orthokinesis where the cells' adaptive reactions to the signal are not stimulated.

Attraction also occurs in populations of *D. discoideum* spreading out into a spatial gradient of cyclic AMP. The mean cell position will initially tend to shift up-gradient. But such attraction is only transitory, and the cells will eventually become randomly distributed, because cell movement is biased-random (Vicker *et al.* 1984). The response consists of klinokinetic and orthokinetic components; but accumulation fails to occur, since the cells are probably always fully adapted to the local cyclic AMP concentration.

These examples lead us to suggest that the accumulation of crawling cells may only occur by either one of two mechanisms: (1) by taxis to soluble temporal signals (with stimulation of the adaptive reactions) or (2) by orthokinesis to adhesive, spatial gradient signals (with no stimulation). In contrast, the accumulation of rapidly swimming organisms, e.g. bacteria, sperm, etc., appears to be driven only by an anisotropic klinokinetic response to soluble, spatial gradient signals and requires self-stimulation of their adaptive reactions.

The specific effects of spatial and temporal signals on neutrophils and amoebae have important consequences. First, although many authors have been perplexed by the 'back of the wave problem' in *D. discoideum* (Parnas & Segel, 1977; Futrelle, 1982; van Haastert, 1983; *inter alia*) it is not a real problem; no paradox actually exists, because amoebae cannot read a spatial gradient whether before or behind them. Their directional responses are confined to a reaction induced by the outward-bound temporal signal alone. Second, investigations of neutrophil behaviour and of the types of immune signals may need to take account of the different cell reactions and various signal forms. *In vivo* it is likely that the signals that are important in the location of inflammatory sites by neutrophils will have a temporal component, especially since neutrophils arrive so early on the scene of inflammatory episodes. Later, once the gradient has stabilized, neutrophil recruitment will perhaps diminish; a feature that would contribute to the gradual restoration of normality. At the very least, greater attention to the detailed characteristics of *in vitro* assay systems is probably necessary if observations are to be interpreted correctly. The practice of some authors of considering all types of directed locomotion and attraction as taxis may be a case in point.

Our results with neutrophils and with *D. discoideum* (Vicker *et al.* 1984) also bear directly on how cells might read morphogen signals in developing systems. The stochastic character of cell reactions to spatial gradients and to the local attractant concentration raises the suspicion that such a signal is in itself incapable of supporting the accurate transmission of positional information to individual cells. In contrast, the nature of the specific cellular reactions to temporal gradients implies that a search for such signals, and perhaps for a corresponding cellular oscillator involved in signal production and interpretation, may be rewarding in several examples of development, e.g. in amphibian somitogenesis (Elsdale & Davidson,

1983) or in the feedback signalling thought to take part in the foundation of the primitive embryonic tissue pattern (see Cooke, 1983).

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