

LEUKOCYTE LOCOMOTION AND CHEMOTAXIS

NEW METHODS FOR EVALUATION, AND DEMONSTRATION OF A CELL-DERIVED CHEMOTACTIC FACTOR*

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The mechanisms that account for accumulation of polymorphonuclear leukocytes (PMNs)¹ at sites of inflammation are not well understood. Presumably there are generated or released at the site, substances that act on PMNs. Such substances might diffuse from the site to establish a gradient affecting the direction of PMN locomotion (chemotaxis), or they might act in other ways, e.g., by stimulating emigration and random locomotion and then immobilizing cells reaching areas where the substances are present in high concentrations.

In recent years numerous interesting and perhaps physiologically important materials have been identified as "chemotactic" for PMNs as a result of studies made employing the Boyden Millipore system (1-6). The material to be tested is placed in the chamber below the Millipore filter (Millipore Corp., Bedford, Mass.), and cells are placed in the upper chamber; after a suitable period of incubation, counts are made of the number of cells that have traversed the filter and are on the bottom side. An increase in this number is taken as an indication of a chemotactic influence. Accelerated emigration of cells through the filter in the Boyden system could reflect true chemotactic influence (i.e., directed rather than random locomotion) or increased rate of locomotion, or both. In some studies attempts have been made to distinguish between chemotaxis and stimulated locomotion by means of additional tests in which the material was placed in the upper chamber or in both chambers (5, 6). The absence of increased cell emigration in these circumstances is then taken to indicate that the positive result obtained when the material is in the lower chamber only is in fact due to chemotaxis, not merely enhanced locomotion. However, even these controls are not entirely adequate to establish the chemotactic basis for the phenomenon, since some substances may stimulate locomotion in low concentrations and inhibit it in high concentrations. Boyden chamber results may also be affected by other factors, such as adhesiveness of cells to the filter material, tortuosity and size of the pore channels, and detachment of cells from the bottom surface (7-9).

We have developed direct morphological methods and have modified the Millipore chamber system to enable better distinction between influences on

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¹ *Abbreviations used in this paper:* AgGG, aggregated gamma globulin; PMN, polymorphonuclear leukocytes.

rate of locomotion and those on direction of PMN movement. These methods have been used to establish that PMNs release under some circumstances substances that stimulate PMN locomotion and also exert a true chemotactic effect on these cells. These actions of cell-derived factors are demonstrable in the absence of serum.

Materials and Methods

Cells.—PMNs from horse and human blood and rabbit peritoneal exudates were used in these studies. Horse blood containing 2.5 mM EDTA stood at room temperature 30 min to allow the spontaneous settling of the red blood cells (RBCs). The white blood cell (WBC)-rich supernate was spun 5 min at 150 g, and the WBC pellet was washed one or more times with saline before resuspending in media at appropriate concentrations. In some experiments the RBCs contaminating the WBC-rich pellet were lysed by reducing the tonicity of the media briefly (30 s) to 30% normal tonicity. Human blood was mixed with an equal volume of 2% dextran-250 in heparinized saline. The RBCs sedimented in about 10 min and the supernate was removed and processed as described for horse cells. Rabbit peritoneal cells (about 95% PMNs) were obtained 4 h after an intraperitoneal injection of 250 ml of 0.1% shellfish glycogen in saline (10).

Essentially pure horse PMNs were obtained by a modification of the albumin gradient method of Bennet and Cohn (11). Cells suspended in 27% albumin were layered over a 1 ml cushion of 29% albumin before centrifugation.

Eosinophils were isolated from horse blood by a modification of the method of Day (12). The eosinophils were pelleted through triosil adjusted to specific gravity of 1.150.

Media and Materials.—Cells were suspended in serum, plasma, or Gey's medium containing 1% horse albumin (Gey-albumin), crystalline human albumin, ovalbumin, casein, or gelatin (Gey-gel). Gamma globulin (human, horse, bovine, or rabbit fraction II; or purified horse gamma globulins) was dissolved in Gey's medium at 20 mg/ml. The solution was passed through a 0.45 μ m Millipore filter and heated 5–10 min in a 70–80°C water bath until the gamma globulin was finely aggregated. The aggregated gamma globulin (AgGG) was pelleted at 900 g for 20 min and washed once with Gey's medium and twice with sterile water before resuspending in sterile water. Other proteins including ovalbumin, gelatin, bovine β -globulin were dissolved or suspended in water or Gey's medium at 20 mg/ml.

Direct Morphological Evaluation of Locomotion and Chemotaxis.—A streak approximately 0.2 \times 4 cm with 1 μ l of AgGG or other protein solution was made with a pipette on a microscope slide and allowed to dry. A small drop of cell suspension (about 4 μ l) was placed on the dry protein streak and a 60 \times 24 mm cover slip was gently lowered over the drop, spreading the fluid smoothly between the slide and the cover slip. The edges of the cover slip were sealed with melted paraffin and Vaseline (1:1). This procedure usually resulted in a monolayer of cells covering an area about 20 \times 20 mm. The thickness of the preparation varied some from area to area but averaged about 10 μ m, which was thin enough to permit good phase-contrast resolution and yet not so thin as to distort the shape of the cells or impair their locomotion. The preparations were observed with a \times 40 objective (diameter of field \sim 0.4 mm) at room temperature or at 37°C, the temperature maintained by a Sage air curtain incubator (Sage Instruments Div., Orion Research Inc., Cambridge, Mass.).

Locomotion was evaluated by determining the percentage of all the PMNs present in a series of \times 40 objective fields displaying a locomoting morphology. At least 100 cells were scored per slide; four or more slides were used to examine each variable.

Chemotaxis was evaluated by determining the direction of locomotion of individual cells as indicated by their morphology, i.e., the front marked by a broad smooth veil (lamellipodium) and the rear marked by constriction into a knoblike tail. Only cells whose morphology allowed classification of their direction of locomotion as either toward or away from the test

line were counted. Again, at least 100 cells were counted per slide and at least four slides were examined for each variable studied. The level of chemotaxis was expressed as the percentage of the cells scored which were oriented toward the test line (see Figs. 1 and 2).

In studies where it was necessary to have cells adherent to the cover slip before the slide preparation was made, 0.1 ml of cells in Gey's medium (5×10^6 cells/ml) were pipetted onto a defined area of a dry cover slip. (The dry cover slip would prevent spreading of a certain amount of fluid.) The cells were allowed to settle and stick to the cover slip in a moist atmosphere at room temperature for about 2 min. The cover slips were then dipped repeatedly in beakers of saline to remove nonadherent cells. (In the absence of protein in the medium most WBCs remained on the cover slip, but most RBCs washed off.) The cover slips were drained rapidly and washed with Gey-gel medium which was also partially drained. The small amount of remaining fluid served to spread the cover slip smoothly over a slide containing a streak of dried protein. Care was taken to prevent the cells from drying at any point during the preparation of these chambers. After the cover slip was in place it was sealed with paraffin and Vaseline.

Millipore Assays of Locomotion and Chemotaxis.—The Millipore chambers were prepared by placing 0.9 ml of medium in the lower compartment of a Sykes-Moore chamber (Bellco Glass Inc., Vineland, N. J.) containing a 25 mm round cover slip and a rubber "O" ring. A 25 mm diameter Millipore filter (3 μ m pore size) was placed over the fluid and held securely in place between the "O" ring and the top metal ring of the chamber, which was screwed down tightly. Any fluid which came through the filter was aspirated before 8×10^6 cells in 1 ml of medium were placed on the top of the filter. The chambers were incubated at 37°C in a 5% CO₂-air incubator. After incubation the top fluid was aspirated and the filters were removed, fixed in buffered formalin, and stained with 8% Giemsa for 1 h. They were then dehydrated and cleared in isopropyl alcohol and xylene. The filters were mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.) and examined with a $\times 40$ objective.

In most cases the filters were scored by measuring with the optical micrometer on the fine focus knob of the microscope the distance from the top of the filter to the furthest plane of focus which contained at least two cells in focus. This measure was taken for five fields across the filter. Duplicate chambers were always run and the data presented are the mean of the two filters.

In certain cases counts were made of the cells on the bottom of the filter or the cells in focus at any given depth into the filter. Again in these cases five fields across the filter were counted and the mean of duplicate filters was used in presenting the data.

Production of "Cell Factor" in a Test Tube.—0.2 ml of AgGG in Gey's medium was placed in a sterile 25 \times 150 mm tube, 0.5 ml sterile saline was added, and the tube was rolled in the horizontal position (at 37°C) until the inner surface of the tube was coated with AgGG. The excess fluid was then drained. Cells were obtained as usual; RBCs were lysed by hypotonic shock and the WBCs were washed three times and suspended in Gey-albumin media. 2 ml of cell suspension were placed in each tube. The tubes were rotated in a horizontal position on a spinner wheel at 35 rpm at 37°C. Control tubes containing Gey's or saline only were rotated similarly. After 1 h the fluid was removed and spun at 2,000 rpm for 20 min. This supernate was used immediately or stored frozen. In some cases the fluid was dialyzed against two changes of 500 ml saline for 18 h followed by dialysis against 150 ml Gey's medium for 4 h.

Freezing and Thawing Cells.—Cells in various media were put through five cycles of freezing in acetone and dry ice and thawing briefly in 37°C water bath. The supernatants after centrifugation (900 g for 20 min) were tested in a Millipore assay.

RESULTS

Studies on PMN Chemotaxis Employing a Direct Morphological Assay System.—The morphologic assay of chemotaxis depended on cell locomotion but was independent of the rate of locomotion. The method made use of the polarity of

locomoting PMNs, which exhibited a broad, thin veil (lamellipodium) at the front and a knoblike tail at the rear. A line of insoluble chemotactic or chemotaxigenic (results in production of chemotactic substance) material was painted on a slide and dried; a small drop of cell suspension was then spread by gently lowering a cover slip over the slide, making a thin preparation (Fig. 1). The preparation was rimmed with a paraffin-Vaseline mixture, and then incubated and observed at intervals by phase-contrast microscopy, or continuously by time-lapse cinematography. Locomoting cells were scored with regard to their

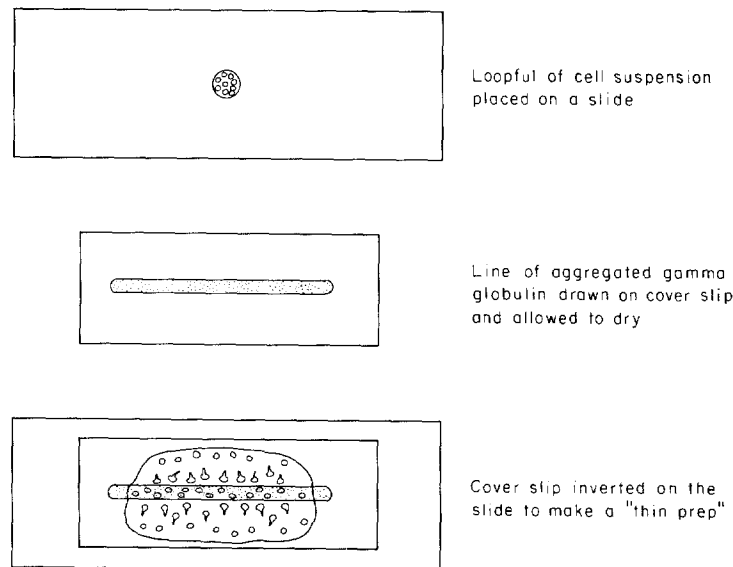


Fig. 1. Procedure for making chemotaxis test system.

orientation in the 180° sectors towards or away from the line of test material (Fig. 2).

To illustrate the test system and its various parameters, we shall present results of observations on horse blood leukocytes at 1×10^8 cells/ml in Gey-gel medium in a thin preparation over a line of horse AgGG. Both locomotion and chemotaxis were stimulated in cells near the AgGG line. This stimulation could be seen within 10 min of making the preparations. By this time some cells lying on the AgGG line were markedly spread, and cells immediately adjacent to either side of the AgGG line were beginning to crawl. As seen in Fig. 3 *a*, with the passage of time, cells further from the line also began to move. Cells further than about 8 mm from the line generally remained rounded and immobile during the standard 3 h observation period in the Gey-gel medium. The stimulation of chemotaxis followed a similar time-course. Fig. 3 *b* shows the development with passage of time of areas with more than 50% of the cells oriented



FIG. 2. Nomarski phase-contrast micrograph of cells locomoting toward a chemotactic line just beyond the top edge of the picture. 17 cells are shown; one is a lymphocyte (*). Of the 16 PMNs, one is not locomoting (*A*) and the direction of two others (*B*) cannot be determined. Two of the remaining PMNs (*C*) are moving toward the lower 180° sector away from the chemotactic stimulus. Thus 15 of 16 PMNs are locomoting, and 11 of the 13 cells displaying clear polarity are oriented toward the upper margin of the field. Approx. $\times 400$.

toward the line. In the field encompassed by a $\times 40$ objective adjacent to the line, the percentage of the cells increased with time from levels near 10% moving and 50% of these oriented toward the line at 10 min, to greater than 90% moving and 90% of these oriented at 30 min. By 2 h, the stimulus had spread so that greater than 90% of the cells five $\times 40$ objective fields out from the line (about 2 mm from the line) were moving and about 70% of these were oriented toward the line (Fig. 4). The cells that had landed on the AgGG had an unusual morphology. They were immobilized and markedly spread, with their nucleus and granules packed into a central area. More than 99% of these spread cells on the AgGG were alive, as determined by trypan blue exclusion.

Human blood leukocytes or rabbit peritoneal exudate PMNs exhibited similar behavior in this test system. Various suspending media were also investigated, including 20% fresh or heated autologous serum, or 1% solutions of horse albumin, human albumin, ovalbumin, gelatin, or casein in Gey's medium. Al-

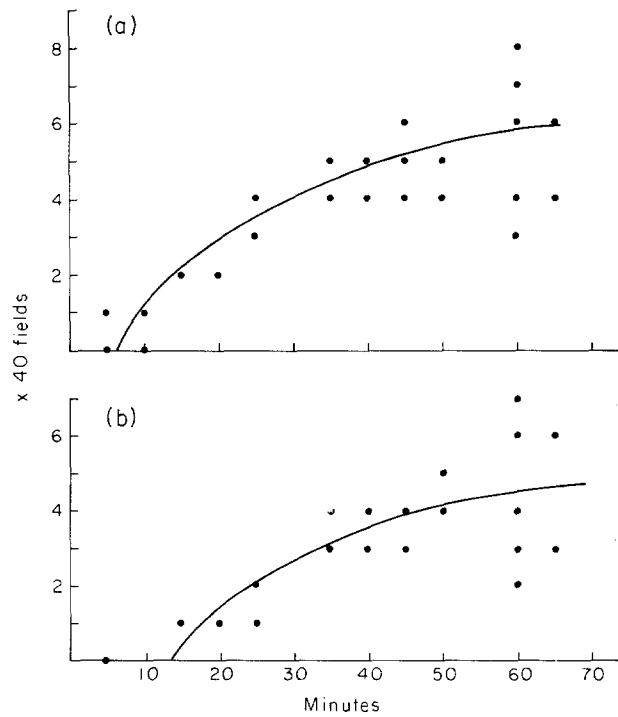


FIG. 3. Ordinate: the distance from the edge of the AgGG line, in $\times 40$ fields, of the field being scored. Abscissa: time in minutes. The points represent the furthest $\times 40$ field in the six slides examined with $\geq 10\%$ of the cells moving (a) or $\geq 50\%$ of the moving cells oriented toward the AgGG line (b) at various times after the thin preparations were made.

though the morphology and the percentage of locomoting cells did vary from one medium to another, the chemotactic response occurred in all instances. The cells responded to lines of aggregated human, bovine, rabbit, or horse gamma globulins, as well as to bovine beta globulin and *Staphylococcus albus*. No response was noted to lines of gelatin, ovalbumin, or lysozyme.

Cell-Derived Nature of the Chemotactic Substance in the Direct Morphological Assay System.—As described above, stimulated locomotion and chemotaxis were seen spreading from the line of AgGG or other material on the slide. It was not possible to determine whether the active substance diffusing outward from the line was derived from an interaction between AgGG and the medium, or whether it was derived from an interaction of the AgGG and those leukocytes that settled by chance on the line. A slight modification of the test system permitted distinction to be made between these two possibilities. The streak of AgGG was made diagonally on the slide as shown in Fig. 5 and the cover slip was lowered on the slide such that in some areas cells would be in contact with the AgGG and in some areas cells would be adjacent to the AgGG, but not on it.

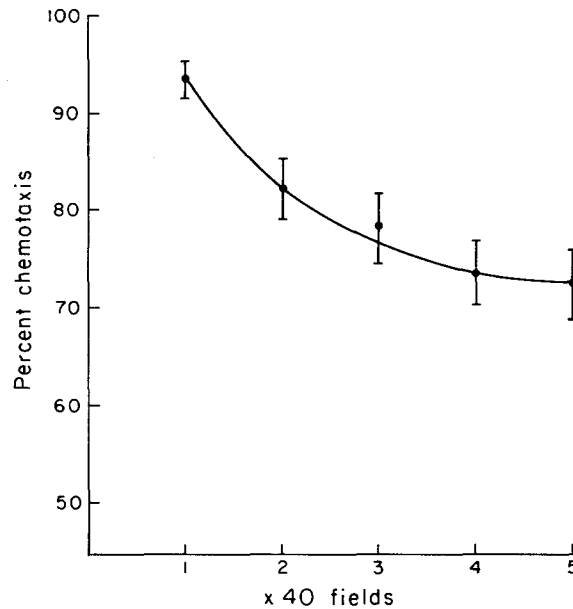
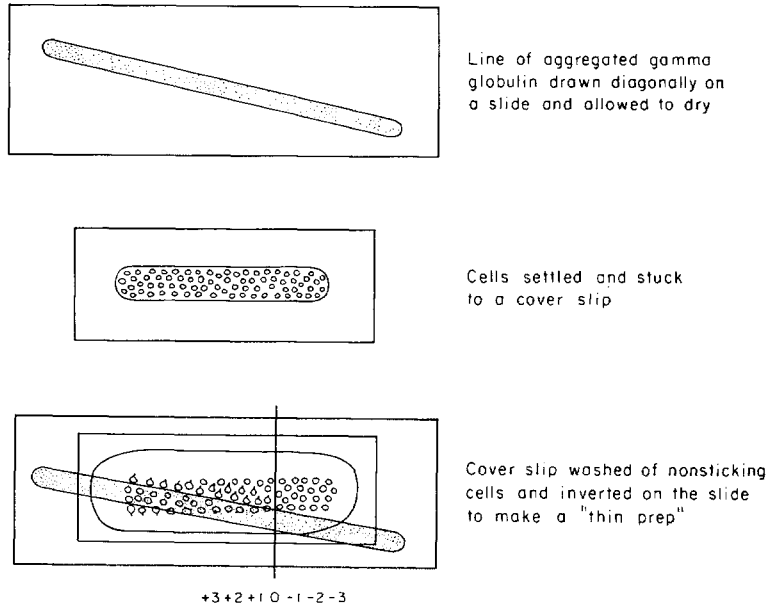


FIG. 4. Ordinate: percent of cells oriented toward the AgGG line. Abscissa: number of $\times 40$ fields from the edge of the AgGG line to the field being scored. The data are compiled from 16 slides analyzed between 30 min and 2 h. The mean \pm SEM are shown.

In regions where cells were in contact with the AgGG, cells adjacent to the AgGG line began to move and orient toward the line. In contrast, in regions where no cells had landed on the AgGG, cells adjacent to the AgGG line generally remained rounded and immotile. Fig. 5 presents the observations made on 12 such preparations.

The experiments reported above utilized horse blood leukocytes. Although approximately 75% of these cells were neutrophils, it was possible that another cell type was responsible for generation of the chemotactic factor. Horse leukocytes were separated into PMN-rich (90% PMNs) and lymphocyte-rich (80% lymphocytes and monocytes) fractions by a modification of the method of Bennet and Cohn (11). Since response of PMNs was being assayed, it was essential to have some PMNs in all populations tested. As shown in Fig. 6, in a number of tests with varying mixtures of two cell preparations there was good correlation of the chemotaxis with the total cell density, and an even better correlation with the concentration of PMNs, whereas there was little correspondence between the PMN chemotaxis and the concentration of mononuclear cells in the population. These results indicated that PMNs were primarily responsible for producing some factor that stimulated motility and chemotaxis. However, since the percent of lymphocytes and monocytes in the populations tested was always 50% or less, it was not possible to conclude that these cells were in-



	+	-
Slides with cells moving well (25%)	10	1
Slides with cells moving slightly	2	3
Slides with no cells moving	0	8
Mean chemotaxis of the moving cells	79%	54%

FIG. 5. 12 preparations with intersecting AgGG lines and cell populations were scored for mobility and chemotaxis in the $\times 40$ fields two fields out (0.8 mm) from the AgGG line and in the first through fourth fields (1.6 mm) to either side of the intersection marked field 0 on the diagram. On the side of the intersection where cells had landed on the AgGG (marked + on the diagram) the cells in the fields being scored moved well and displayed a chemotactic response. In contrast, there was little cell movement and no evident chemotaxis toward the AgGG line which had no cells on it [(-) side].

capable of producing a factor chemotactic for PMNs in this system. Using a triosil separation method (12) the role of eosinophils in PMN chemotaxis was also examined. Populations with less than 0.1% eosinophils showed excellent chemotaxis of PMNs. Populations enriched in eosinophils (20–40%) were used to determine that these cells responded to the chemotactic influences in a manner similar to that of neutrophil PMNs. In the regions adjacent to the AgGG line, 85% of the eosinophils oriented toward the line.

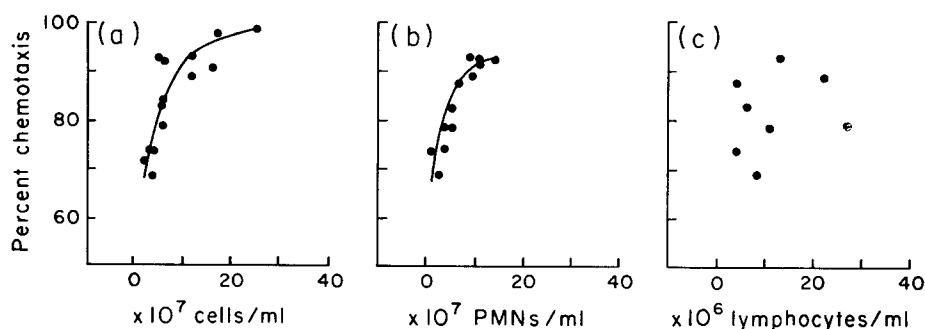


FIG. 6. Ordinate: percent of PMNs oriented towards the AgGG line. Abscissa: concentrations of WBCs (a), PMNs (b), or lymphocytes (c) in the cell suspension used to make the thin preparations. Each point represents the percent chemotaxis of at least 100 cells scored.

Studies on PMN Locomotion and Chemotaxis Using the Millipore Chamber Assay.—The Boyden system employs two chambers separated by a Millipore filter. The putative chemotactic factor is placed in the lower chamber, and a cell suspension is placed in the upper chamber. After an appropriate incubation period, chemotaxis is assayed by counting the PMNs which have moved through the Millipore filter and are found on the bottom side of the filter. The pores of the filter, 3 μm in diameter, are large enough to allow the cells to crawl through, but small enough to prevent cells from falling in passively. As mentioned earlier, the interpretation of the results in terms of chemotaxis is complicated by effects of the materials being tested on the rate of random locomotion. In addition, the parameter usually measured, i.e., the number of cells on the bottom of the filter, is not reproducible, partly because cells may fall off the lower surface of the filter with prolonged incubation (8, 9).

To circumvent some of these problems, we modified the method as follows. Cell locomotion (random locomotion) was assayed by measuring the distance into the filter that the front of the cell population had moved when the same material was present homogeneously on both sides of the filter. Thus, instead of incubating the cells on the filters long enough for them to crawl all the way through the filter, the incubation was stopped when the front cells were well into the filter, but not yet at the far side. The filters were fixed, stained, and cleared with xylene. Using a $\times 40$ objective, the distance from the focal plane on the top of the filter to the furthest two cells in one focal plane was then measured with the micrometer on the fine focus. This measure was taken for five $\times 40$ fields across each of two duplicate filters and the mean of these 10 measurements was calculated.

The time-course of locomotion in the absence of a gradient is shown in Fig. 7. The distance moved increased nearly linearly with time between 10 and 60 min. The time-course had a similar pattern whether the cells were moving sluggishly in serum-free media or were moving rapidly in media containing 10% serum. In

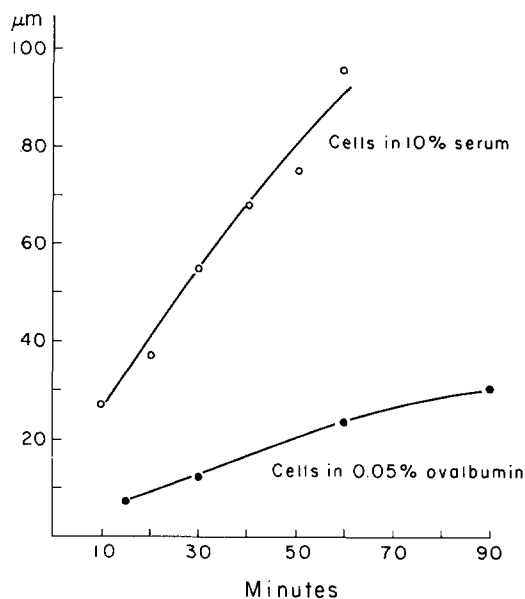


FIG. 7. Ordinate: distance (microns) into the filter of the cell front (two PMNs in one focal plane). Abscissa: time of incubation of the Millipore filter chambers (minutes). Horse cells were incubated in the Millipore chambers for varying lengths of time. In each instance, the medium used to suspend the cells (either 10% horse serum in Gey's medium, or 0.05% ovalbumin in Gey's medium) was also present below the Millipore filter.

contrast, the number of cells on the bottom of the filter, which is the parameter usually measured, did not increase continuously with time, but reached a peak and then declined (Fig. 8). By summing the cells on the bottom of the filter with those which had fallen off the filter, a curve increasing continuously with time was obtained.

Duplicate filters were routinely run as a check of the reproducibility of the method. The distance moved in each of 100 duplicates was examined; the individual filters differed from the duplicate mean by an average of 8%. The reliability of the assay was similar for horse, human, and rabbit cells. In contrast, an examination of the reliability of scoring the number of cells on the bottom of the filter indicated that the mean differed from the individual filters by an average of 23%. The resolution of the method, that is the ability to detect differences in rates of locomotion, was of course greatest when nearly the full depth of the filter was utilized. Differences in slowly moving populations could be resolved by running those assays longer.

It was important to know if the distance moved by the two front cells was a true indication of the movement of the whole population or whether those cells represented a subgroup within the population. To examine this question, the distribution of cells in the filter was studied at various times during the incuba-

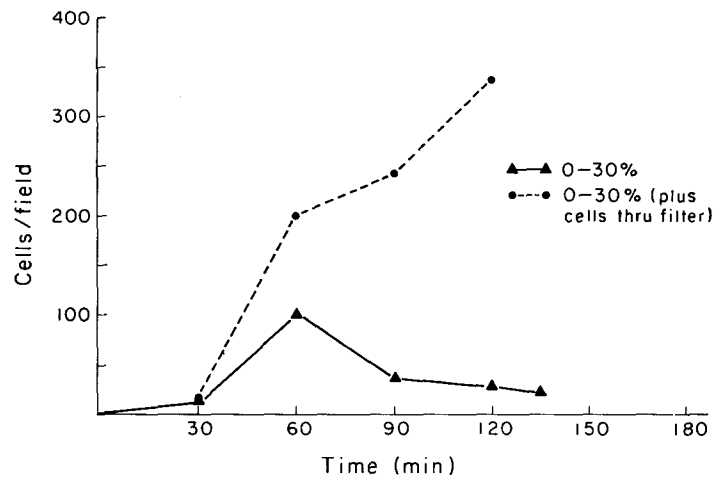


FIG. 8. Ordinate: number of cells per $\times 40$ field on the bottom of the Millipore filter (\blacktriangle), or the total number of cells per $\times 40$ field on the bottom of the filter plus those on the cover slip at the bottom of the chamber (\bullet). Abscissa: time of incubation of Millipore filter chambers (minutes). Horse cells in Gey's medium were placed on top of the filter. 30% horse serum in Gey's medium was present below the filter. The diameter of the cover slip at the bottom of the chamber (where cells which had traversed and fallen off the filter landed) was the same as the diameter of the Millipore filter (i.e., 25 mm).

tion. The suspending media for these studies was Gey's containing 4% normal horse serum. The distribution of PMNs in the filter after various incubation times was similar to that which would be expected from a uniform population of particles exhibiting a random-walk type of behavior (Fig. 9). This was most clearly seen in the characteristic linear relationship of a random-walk type process between the log of the number of particles (in this case PMNs) at given distances vs. the square of the distance (Fig. 10). The experimentally determined distance to the front two cells fell near the line determined by the least squares method from the total population distribution; thus the two-cell measurement was representative of the whole population. Similar results were obtained after 10, 20, 30, 40, and 50 min incubation. Additional experiments demonstrated that the variations in the distance measured with changes in cell number was also characteristic of that which would be expected of a randomly moving population. Increasing the cell concentration from 1×10^6 to 1×10^7 per ml resulted in about a twofold increase in the distance measure in a 60 min assay in 25% serum. Thus the distance to the front two cells per $\times 40$ field seemed to be a relatively sensitive and reproducible indication of the rate of locomotion of the PMN population.

Having a reliable Millipore assay of random locomotion, the effects of different concentrations of a given substance on locomotion could be investigated. Then the directional response of the cells could be tested in environments of

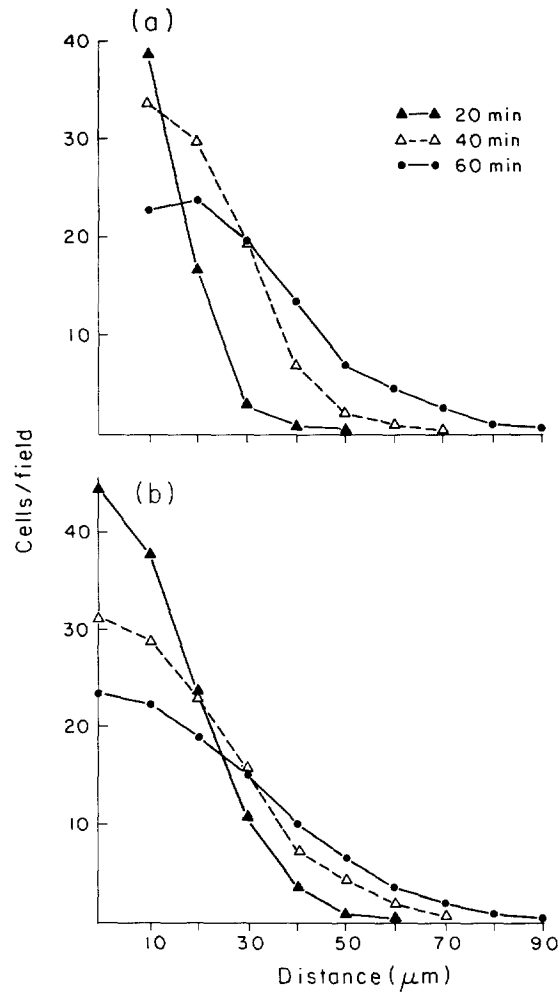


FIG. 9. Ordinate: number of cells per one-fourth of a $\times 40$ field. Abscissa: microns from the top of the filter to the level being counted. (a) 6×10^6 horse WBCs in 4% normal horse serum were placed on top of a Millipore filter. 4% serum was also present below the filter. Chambers were incubated for 20, 40, or 60 min. The number of cells in focus per one-fourth of a $\times 40$ field were then counted at successive planes, each $10 \mu\text{m}$ from the top of the filter. Each point represents the mean of 10 fields counted, 5 on each of 2 duplicate filters. (b) The number of particles at various distances from the origin calculated for a population of 1×10^8 particles per one-fourth field (C_0) and a diffusion coefficient (D) of $8 \mu\text{m}^2/\text{min}$. The calculations were made from the equation for particles moving out of a thin layer (13). The right-hand side of the equation has been multiplied by 2 since in the situations under consideration the particles are only moving in one direction from the origin.

$$c = \frac{C_0 dx}{\sqrt{\pi D t}} e^{(-x^2/4Dt)}$$

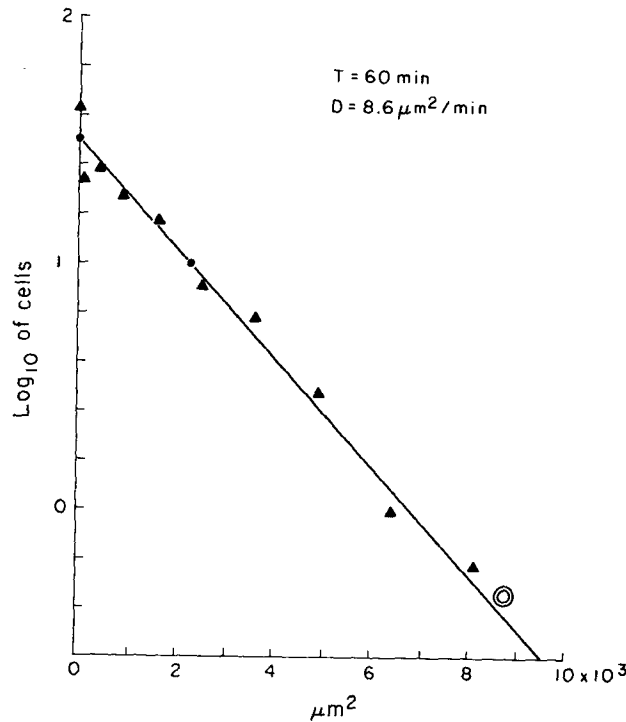


FIG. 10. Ordinate: \log_{10} of the number of cells per one-fourth of a $\times 40$ field after a 60 min incubation. Abscissa: square of the distance from the top of the filter to the field counted (square microns). The data are taken from the experiment described in Fig. 11. Each (\blacktriangle) is the mean of five determinations of the number of cells in focus per one-fourth of a $\times 40$ field at successive $10 \mu\text{m}$ distances into the filter. (\odot) is the distance to the front two cells per $\times 40$ field for this filter. The D was calculated from the slope of the line on this graph. The line was drawn by the method of least squares (\bullet).

known influence on the rates of random locomotion. One could predict how far, on the basis of rates of random locomotion alone, the cells would move in any given gradient. The prediction could be merely comparative, i.e., they should move further in the gradient $x \rightarrow y$ than in $x \rightarrow x$, if the random mobility in y is greater than in x . It would also be possible to predict more quantitatively how far the cells would be expected to move. In any event, there would be evidence for a chemotactic factor if the actual movement of cells in a positive gradient of a test material were greater than that predicted on the basis of random locomotion.

Demonstration Using the Millipore Chamber Assay of a Leukocyte-Derived Factor Stimulating Locomotion and Chemotaxis.—When leukocytes were incubated in tubes that had been coated with AgGG, there appeared in the medium a substance capable of stimulating leukocyte locomotion as measured by the

Millipore chamber method. This activity did not appear in control specimens incubated in similar fashion but containing no leukocytes. The stimulatory effect was dependent on the concentration of active supernate placed in the Millipore chambers. As is illustrated in Fig. 11, increasing concentrations of the supernate up to 10% led to striking increase in the rate of locomotion into the filter; however, concentrations of the supernate higher than 10% resulted in progressively less stimulation of cell migration. The stimulation of locomotion by low concentrations of cell factor was reversible, but the lessened stimulation (relative inhibition) associated with high concentrations of cell factor was not completely reversible. The stimulatory effect on locomotion was quite rapid;

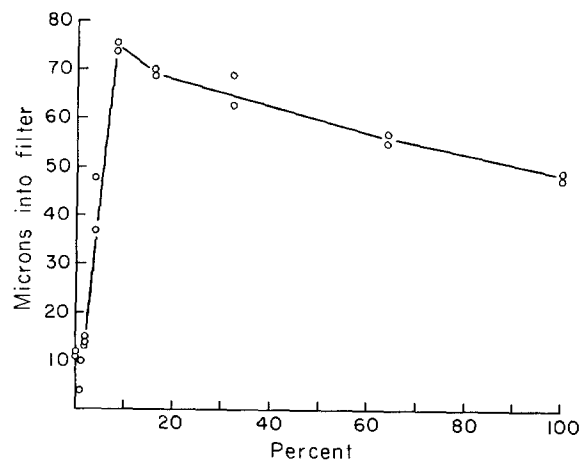


FIG. 11. Ordinate: microns moved into filter by the cell front during 30 min incubation. Abscissa: concentration of cell supernate present both above and below the Millipore filter. The supernate was obtained after 1.5×10^7 horse leukocytes/ml were rotated at 37°C for 1 h in Gey-albumin medium in a tube coated with AgGG.

preincubation of cells for 0, 10, or 30 min in a medium containing 12% active supernate did not alter the stimulated locomotion in a 30 min assay in the same medium. The relative inhibitory effect of high concentrations of active supernate increased with time. Cells preincubated with 50% active supernate for 30 min moved less well in a test medium containing the same supernate than did cells not preincubated.

In order to determine whether the supernates contained chemotactic activity as well as the capacity for stimulation of locomotion, different concentrations of supernate were placed above and below the filter so that a gradient was established across the filter, and measurements of cell movement were made to determine if there were effects greater than those to be expected on the basis of those on rates of locomotion alone. The results shown in Tables I and II suggest the presence of a chemotactic factor in the leukocyte supernates. For example,

TABLE I
Mean Micron Penetration into the Filter, Observed

% supernate above filter	% supernate below filter				
	1	4	8	40	80
1	26	47	85	121	
4		56	100	102	
8	35	79	84	95	71
40		60		76	
80			66		56

Mean distance of five fields on each of two filters to the front two PMNs in one focal plane during a 30 min incubation as influenced by various concentrations of supernate above and below the filter.

TABLE II
Calculated Micron Penetration to be Expected on the Basis of Effects on Locomotion Alone

% supernate above filter	% supernate below filter				
	1	4	8	40	80
1	26*	31	37	60‡	
4		56*	64	79‡	
8	71	77	84*	82‡	78‡
40		79		76*	
80			65		56*

Distance to the front two PMNs as calculated for a randomly moving population. The calculations described in the Appendix were based on the observed rates of locomotion in the absence of a gradient (marked with asterisk).

A dependent *t* test showed the results to differ in the direction of positive chemotaxis from that of random locomotion with a $P < 0.03$. Circled values not used in the *t* test since the inhibitory effect on locomotion of high concentrations of supernate are only partially reversible.

‡ Scores were increased by 15 before used in the *t* test to correct maximally for any error due to the slow time-course of inhibition of concentrations of supernate greater than 40%.

in this experiment cells in 8% supernate would be expected to move less rapidly toward 40% than toward 8% supernate, since (see Fig. 11) locomotion was stimulated less in 40% than in 8% supernate, whereas in fact cells moved more rapidly (95 μm) towards this concentration gradient of supernate from 8 to 40% than they did in the absence of a gradient (84 μm with 8% above and below the filter). Similarly, cells in 1% supernate moved equally well towards 8% supernate as did cells in 8% move towards 8% supernate. Thus, in both of these cases, cells moving up a concentration gradient moved further than expected on the basis of random locomotion alone. Cells in 8% supernate moving toward 1%, i.e. moving down a concentration gradient, moved only 35 μm , less than one-half the distance moved by cells in 8% toward 8% or cells in 1% toward 8%.

This decrease in distance moved occurred despite the fact that the cells were highly stimulated to move at the beginning of this assay. The instances in which the cells moved the furthest were generally those in which the largest positive gradient existed across the filter. For instance, cells in 1% supernate moving toward 40% supernate penetrated a distance of 121 μm . In a few cases, there was no evidence of chemotaxis. For example, cells in 8% supernate moving toward 80% supernate penetrated only 71 μm into the filter, less than the penetration of cells in 8% supernate moving toward 4%, 8%, or 40% supernates.

If the effects of the materials on the cells were rapid and reversible, it would be possible to estimate how far the front two cells would be expected to move if they were moving in a random-walk process in which the only variable was the rate of random locomotion. For such a calculation the gradient across the filter would be assumed to be linear, stable, and extending just across the filter.² (Calculations are described in the Appendix.) These predicted values are shown in Table II. Comparison of these values with the experimental values suggested that the cell supernates had exerted chemotactic influence, i.e. the cells in general moved further in a positive gradient, and less far in a negative gradient, than was to be expected on the basis of effects on random locomotion alone. Additional experiments gave similar results.

Evidence in support of a chemotactic response can also be gathered from studies of the distribution of the cell population throughout the filter. In the case of random locomotion, highly stimulated or not, the peak of cell concentration will remain at the origin until a uniform distribution has been achieved. In contrast, if a true chemotactic influence exists, the cells would be expected to move en masse towards the source or the gradient, and this en masse movement combined with diffusion results in the peak of cell concentration moving into the filter. This criterion was used to confirm the true chemotactic influence of the cell-derived factor in our tests (see Fig. 12).

The characteristics of the cell-derived factor, and the requirements for its generation and action have been only partially defined, but the following statements can be made at present. The stimulatory activity in the supernate of cells rotated in an AgGG-coated tube was not decreased by dialysis for over 18 h, or by heating at 56°C for 30 min or at 80°C for 10 min. Leukocytes rotated in clean tubes released some activity, but this activity was never as high as that produced in AgGG-coated tubes. A material with similar stimulatory activity could be obtained by repeatedly freezing and thawing horse leukocytes. Stimu-

² The time for a linear gradient to be 99% complete across the 150 μm Millipore filter if the substance in question were the size of albumin was calculated to be 45 s (13), an insignificant time relative to the 30 min assay. The stability of the gradient was validated by experiments showing that a gradient of trypan blue dye across the Millipore in a chamber decreased less than 10% during a 30 min incubation. Since there was no stirring of the medium on either side of the filter, the gradient was probably not limited to the depth of the filter, but could have extended out slightly into the medium on either side.

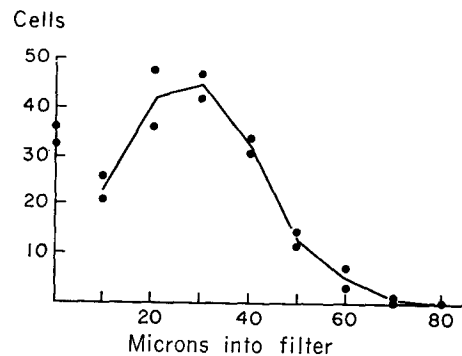


FIG. 12. Ordinate: number of cells in focus in one-fourth of a $\times 40$ field. Abscissa: microns into the filter to the field counted. Cells in Gey's medium were placed on top of the filter and a 50% dilution of cell supernate placed below the filter. Chambers were incubated 30 min. The supernate was made in Gey-albumin as described in Fig. 11.

latory activity was present in supernates of rotated cells when the suspending medium was Gey's plus 1% gelatin, horse albumin, or human albumin, whereas in supernates from cells rotated in protein free Gey's there was no activity. The production or release of an active factor apparently required glycolytic energy, since 10^{-3} M deoxyglucose or 10^{-4} M iodoacetate inhibited the appearance of an active material. (Before testing these supernates in the Millipore, they were dialyzed thoroughly to free them of the inhibitors.) When the blood leukocytes were separated into PMN-rich (90%) and lymphocyte-rich (80%) populations, the PMNs produced more active supernates, but there was more activity produced from the lymphocyte-rich population than could be accounted for by its PMN content. Furthermore, mouse peritoneal cells, almost entirely lymphocytes and macrophages, were also capable of producing active supernates.

DISCUSSION

The Slide-Cover Slip Method for Measurements of PMN Locomotion and Chemotactic Influences.—Locomotion and chemotaxis of ameboid cells have been studied for over 100 years, employing a wide variety of methods. Essentially these techniques may be divided into two categories: (a) those involving direct microscopic observation of individual cells, or (b) those involving measurement of the behavior of a cell population. Many of the procedures have been inadequately investigated for possible artifactual errors; the pitfalls have been discussed in detail in the review of Harris (15).

The direct observation method used by us differs in important details from that employed by Harris or others. Rather than tracing a track, we have used to evaluate the direction of locomotion of PMNs the distinct polarity of the cells, with a broad, advancing lamellipodium and a tail-like constriction at the rear, thus making it possible readily to measure the levels of orientation at any given

position and time. For routine evaluation no photographic processing was necessary; the cells could be scored directly under the microscope. Time-lapse cinematography confirmed that the cell polarity and orientation was a true reflection of direction of locomotion.

Previous studies have suggested that rates of locomotion (16, 17) are not altered by a chemotactic stimulus. In some of these instances the conditions employed led to maximal or near maximal rates of locomotion, thus masking possible stimulation of locomotion by chemotactic factors added. In addition, cells somewhat distant from a chemotactic source may be stimulated to move by a chemotactic material which is no longer in a gradient sufficient to induce directed locomotion. Although rates of locomotion were not examined in our thin preparations, we did note a higher percentage of cells locomoting in association with chemotaxis. Perhaps some materials that stimulate chemotaxis also stimulate locomotion, at least in certain concentration ranges.

The degree of chemotaxis decreased as we examined $\times 40$ objective fields further from the line source. The failure to observe this decrease in an earlier study (17) was perhaps due to the fact that all observations were made within $500 \mu\text{m}$ ($\sim 1 \times 16$ objective field) from the source.

It is difficult or impossible to establish the origin and nature of the true chemotactic factor in the usual complex test system containing a stimulating material, various medium components, and a mixture of cell types. Does a material that stimulates chemotaxis do so directly, or does it need to interact first with the medium, or does the chemotactic factor derive from cells that encounter the material by chance and are as a result of this encounter stimulated to release a chemical messenger to call neighboring cells to the scene? A simple modification of the direct method used here enabled us to answer this question. The slide and cover slip preparations were made in such a way that lines of stimulating material (e.g., AgGG) and of adherent PMN, were crossed at a gentle angle. Examination of areas near the intersection (see Fig. 5) allowed observation to be made of PMN locomotion and chemotaxis adjacent to stimulating material admixed with cells, as compared with PMNs situated equidistant from stimulating material devoid of cells. The results indicated clearly that chemotactic factor was generated only when AgGG and leukocytes had come into contact. Further studies of this type established that the cells primarily responsible for generation of chemotactic material were PMN leukocytes, not mononuclear ones. Both neutrophil and eosinophil PMNs exhibited chemotactic responses.

The slide-cover slip method is simple and reliable, but there are limitations and important technical factors. The preparations were made as thin as practicable (about $10 \mu\text{m}$) without impeding cell movement, in order to minimize fluid currents and to prevent rapid dilution of the gradient. Chemotactic influences were detectable in a band (approximately 3 mm) adjacent to the line of stimulating material.

The media employed in most of our experiments contained no source of complement or other serum factors, and the cells were washed repeatedly to remove serum factors as completely as possible. It is, of course, well known that AgGG or other factors can interact with complement to generate chemotactic factors (1, 2, 4). Chemotactic factors originating from a serum source might well have masked and rendered difficult or impossible the demonstration of the cell-derived factor. One further advantage of the serum-free media was notable. PMNs exhibited but little spontaneous locomotion in these media, and the observations indicated clearly that all experimental conditions that stimulated chemotaxis also stimulated locomotion, a finding of relevance to the interpretation of results using the Millipore chamber method, as discussed below.

The Millipore Chamber Method for Assessing PMN Locomotion and Chemotaxis.—The direct methods for measuring locomotion and chemotaxis, though simple and reliable, are limited in flexibility and tend to be time consuming since a large number of cells must be observed individually to obtain statistically significant results. Studies on movement of cell populations—in tissue culture explants, in capillary tubes, or in the Millipore chamber—are thus to be preferred from some viewpoints. The Boyden Millipore chamber (1) offers great utility for studies on cell locomotion and chemotaxis, and most recent work has employed some modification of this system. We have pointed out above the inaccuracies attendant on use as the endpoint of total number of cells migrating through the Millipore filter and residing on the opposite surface. The measurement of the distance moved into the filter by the advancing cell front is much more reproducible. The general behavior of PMNs in the Millipore follows closely that expected of a random-walk diffusion process. The position of the cell front, as determined by location of the deepest level containing two cells in a focal plane of a high power field, was shown to be a valid index of the behavior of the total cell population. Knowledge of the distance traveled by the cell front and the total number of cells on the filter at the outset allowed calculation of the total population distribution for a standard diffusion process; these calculated values agreed well with experimental determinations of the cell distribution in the filter.

In any of the methods for observing movement of cell populations the results are determined by three distinct parameters, namely the percentage of cells locomoting, the rate of cell locomotion, and the random or direct nature of this locomotion. The measure of the distance to the front two cells is relatively insensitive to changes in the number of locomoting cells; a 10-fold increase in cell number was found only to double the distance measure. This is in contrast to the number of cells on the bottom of the filter which would be expected to be directly proportional to the number of cells moving. Under most circumstances it is difficult or impossible to distinguish in the Millipore system between effects on locomotion or chemotaxis. In some instances chemotactic factors at a variety of concentrations have been shown to affect rate of locomotion only

slightly (5), but this property of a substance cannot be assumed. Particularly important is the possibility that high concentrations of certain factors may inhibit locomotion (18). Others have attempted to distinguish between effects on locomotion and those on chemotaxis by placing the active material on both sides of the filter. We have extended this approach by measuring effects on locomotion of various concentrations of stimulating materials, and then defining chemotaxis as those instances in which cells moved into the filter in response to a positive gradient of active substance more rapidly than would be expected on the basis of effects on locomotion per se. The conclusion that such observation validates a chemotactic response requires that: (a) the stimulatory and/or inhibitory effects of the material are rapid and reversible, or if not so that the deviations due to these factors have been appropriately taken into account; (b) that a sharp peak of stimulatory or inhibitory effects on locomotion has not been missed in the serial dilutions; (c) that a stable gradient of test material extends across the 130 μm of the filter.

Evidence for a Cell-Derived Factor Stimulating Locomotion and Chemotaxis.—Our experiments establish that a factor(s) derived from PMNs can stimulate PMN locomotion and chemotaxis in the absence of serum. The requirement for cells for production of activity in both systems was clearly established. Cells were required to be present on the AgGG line in order to get a chemotactic response in the slide-cover slip preparations. In a rotating AgGG-coated tube, cell suspensions, but not media alone, produced an active soluble material. In both test systems the response was proportional to the cell concentration, and there was a correlation in particular to the PMN concentration rather than to the concentration of mononuclear cells. However, the possibility that lymphocytes or monocytes produced a stimulatory material under these conditions was not ruled out. In fact, some stimulatory activity was obtained by rotating in AgGG-coated tubes washed mouse peritoneal cells which are primarily monocytes and lymphocytes.

There seemed not to be a rigid requirement for AgGG as the stimulating material. In the slide-cover slip preparations lines of dried bovine beta globulin or *S. albus* also served as chemotactic or chemotaxigenic agents. Cells rotated in clean tubes or cells repeatedly frozen and thawed produced some activity demonstrable in the Millipore system. The production or release of chemotactic activity by cells rotated in an AgGG-coated tube required glycolysis. The active material was heat stable and nondialyzable. Only these preliminary studies have been done on the nature of this material and the factors determining its production in various cells.

The relationship between the active material described in these experiments and chemotactic materials described by others in the literature is not clear. A number of studies have demonstrated chemotactic activity associated with various complement components (2). Although the chemotactic response reported here occurred in the absence of serum, we could not rule out possible complement components bound to the cells, even after extensive washing. How-

ever, the requirement for glycolysis for the production of the active factor speaks against the involvement of complement or other serum factors on the cell surface.

The production of chemotactic activity in our studies occurred under conditions where the cells might be attempting to phagocytize the AgGG-coated surface ("frustrated" phagocytosis) (19). Under such conditions lysosomal enzymes may be released into the medium. Furthermore, certain lysosomal enzymes have been implicated in the production of chemotactic factors. Yoshinaga et al. (20) reported that lysosomal enzymes could digest Ig and produce a chemotactic material. Such a material could have been generated in our system, but cannot explain all our findings since a chemotactic activity was also produced by exposing cells to a surface coated with beta globulins or staphylococci, or even by simple freezing and thawing of the leukocytes. Ward and Hill (21) have shown that rabbit PMN granules contain a protease capable of activating C-5. However, as mentioned above, there was no obvious source of C-5 in our system, and in addition 0.03 M epsilon aminocaproic acid, which is known to block the activity of the rabbit granule protease, did not inhibit the production of stimulatory activity by horse cells in an AgGG-coated tube.

Many of the reports of "cell-derived chemotactic factor(s)" are based on observations made in the presence of plasma or serum (22, 23), and activation or alteration of complement or other serum factors may thus have occurred. However, Borel et al. (24, 25) reported release during a 2 h incubation of rabbit peritoneal PMNs in a serum-free medium of a factor that is active in stimulating the movement of PMNs through a Millipore in the absence of serum. The production of this factor was enhanced by the presence of gamma globulin complexes, as was also the case in our system. Borel and colleagues observed that 40% of their cells were trypan blue positive after incubation with AgGG, and in cell fractionation studies they observed the activity in a postgranule supernate. The factor was obtained from PMNs and in lesser amounts from peritoneal macrophages, but not from lymphocytes of mesenteric lymph nodes. Thus they suggested that the active material was a cytoplasmic constituent released by the dead cells, in contrast to our observations of release from viable cells.

Phelps (6, 26) reported the release by cells in contact with urate crystals, but not soluble urate, of a material stimulating locomotion of cells through a Millipore filter in the absence of serum. He also showed that this material did not stimulate locomotion when placed both above and below the filter, thus suggesting that a chemotactic activity was present. It is possible that the activity described by Borel and Phelps is due to a material similar or identical with the one we have investigated.

SUMMARY

Polymorphonuclear leukocyte (PMN) locomotion and chemotaxis have been evaluated by direct microscopic observation of individual cells in thin slide-cover slip preparations, and also by observations on populations of cells mi-

grating into a Millipore filter. The direct microscopic method used the polarity of the locomoting PMNs (broad, advancing lamellipodium and knoblike constriction at the rear) to record the direction of movement. The Boyden chamber Millipore assay was made more reliable by following the front of cells advancing into the filter, rather than counting the number of cells on the lower filter surface. Special modifications of the Millipore assay were necessary in order to distinguish between influences on rate of locomotion and true chemotaxis.

In both systems the results indicate that under certain conditions leukocytes, and in particular PMNs, release into the medium a factor stimulating locomotion and exerting chemotactic action on PMNs in the vicinity. This cell-derived factor appears not to require serum factors for its release or action.

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APPENDIX

Method for Calculating Distances Cells Are Expected to Move on the Basis of Locomotion Alone (No Chemotaxis)

Assumptions.—

- (a) A linear gradient extends across the 130 μm filter.
- (b) Acceleration of cells between two known velocities is constant.
- (c) Movement of the front two cells of the population can be considered as a single particle moving with a velocity which is a function of the concentration of stimulatory material.

In these experiments (a) the time for all assays was 30 min (1 unit time), (b) the "velocity" in a given concentration of serum (or supernate) was determined experimentally as the distance to the front two cells after incubation in that concentration for 1 unit time, (c) the distance into the filter from the top of the filter to the first serum concentration for which a velocity is known is d_1 ; d_2 is the distance in the filter between that serum concentration and the next serum concentration with a known velocity; d_3 , etc.

For example, in a test of cells moving from 10% serum toward 50% serum placed below the filter for which we know velocities in 10% = 84 μm = V_0 , 30% = 73 μm = V_1 , and 50% = 60 μm = V_2 , d_1 (distance from the top of the filter to the place in the filter where the serum concentration is 30%) will be one-half the way through the 130 μm filter or 65.5 μm into the filter. (Since we have assumed a linear gradient of serum across the filter, the distance to 30% serum which is one-half the way between 10% and 50% serum will be one-half the way through the filter.)

To complete the calculation of how far the front two cells would be expected to move in this serum gradient:

$$d_1 \quad 65.5 \mu\text{m}.$$

$$V_0 \quad 84 \mu\text{m}/\text{unit time}.$$

- V_1 73 $\mu\text{m}/\text{unit time}$.
 V_2 60 $\mu\text{m}/\text{unit time}$.
 \bar{V}_1 The mean velocity over distance $d_1 = (V_0 + V_1)/2 = 78.5 \mu\text{m}/\text{unit time}$.
 T_1 The time to move from the top of the filter to $d_1 = (d_1)/(\bar{V}_1) = 0.835$ unit time.
 d_2 The distance from 30% serum to 50% serum = 65.5 μm .
 \bar{V}_2 $(V_1 + V_2)/2 = 66.5 \mu\text{m}/\text{unit time}$.
 T_2 $(d_2)/(\bar{V}_2) = 0.986$ unit time.

Since $T_1 + T_2$ is greater than 1 unit time the front two cells will not get all the way across d_2 . To calculate how far into d_2 they will get in 1 unit time:

$$a_2 \text{ (acceleration over } d_2) = \frac{V_2 - V_1}{T_2} = -13.2 \mu\text{m}/(\text{unit time})^2,$$

T_f = time remaining after cells have crossed $d_1 = 1 - T_1 = 0.165$ unit time.
 Distance into $d_2 = V_1(T_f) + a_2/2(T_f)^2$. The total distance the front cells would be expected to move would be

$$d_t = \bar{V}_1(T_1) + V_1(T_f) + \frac{a_2}{2}(T_f)^2; \bar{V}_1(T_1) = d_1.$$

Thus

$$d_t = 65.5 \mu\text{m} + (73 \mu\text{m}/\text{unit time})(0.165 \text{ unit time}) + \frac{-13.2 \mu\text{m}/(\text{unit time})^2}{2} (0.165)^2,$$

$$d_t = 77 \mu\text{m}.$$

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