Immunology 1978 35 539

Use of leucocyte migration under agarose to study spontaneous and directed locomotion of leucocytes

H. REPO, ANJA A. I. KOSTIALA & T. U. KOSUNEN *Department of Bacteriology and Immunology, University of Helsinki, Helsinki, Finland*

Received 1 *January* 1978; *accepted for publication* 23 *January* 1978

Summary. Three different cell attractants, together with the parallel use of the leucocyte migration agarose test (LMAT) and the leading front modification (LFM) of the Boyden chamber technique, were employed in studying whether the maximal migration of normal human polymorphonuclear leucocytes (PMNs) is higher toward an attractant (chemotaxis) than in the same attractant incorporated in the culture media (chemokinesis). Using LMAT, the maximal migration distance toward zymosan activated serum (ZAS) was found to be significantly longer than that under agarose mixed with ZAS, thus indicating a chemotactic effect exerted by ZAS. When bacterial culture filtrate (BCF) and casein were used as attractants, the corresponding difference was not significant, implying that the stimulatory effect of these substances on cell migration could be explained by increased random locomotion (chemokinesis) alone. In LFM, the migration rate was significantly higher along a casein gradient than without a gradient. Using ZAS, however, only chemokinesis could be demonstrated. BCF was found to attract PMNs into membrane filters only in the presence of human serum albumin. These observations give credence to the view that both LMAT and LFM are applicable to the *in vitro* assessment of chemotaxis and chemokinesis but the attractant of choice for this is different in each of the two methods.

Correspondence: Dr Heikki Repo, Department of Bacteriology and Immunology, University of Helsinki, Haartmaninkatu 3, 00290 Helsinki 29, Finland.

0019-2805/78/0900-0539 $02.00

INTRODUCTION

Chemotaxis is considered to be positive if cells migrate toward the site of the highest attractant concentration and negative if they migrate away from it (McCutcheon, 1946). Enhanced random locomotion of cells induced by chemical substances in their environment, i.e. chemokinesis (for definition of the terms related to locomotion of PMNs, see Keller, Wilkinson, Abercrombie, Becker, Hirsch, Miller, Scottramsey & Zigmond, 1977), is well documented and has been demonstrated by a membrane filter technique (Zigmond & Hirsch, 1973; Wilkinson, 1974a), by photographing paths of individual cells (Keller & Sorkin, 1966) and by the buffy coat assay of Meier (Meier, Desaulles & Schär, 1955), in which the cells migrate out of an explant of clotted plasma.

The membrane filter technique devised by Boyden (1962) and since then modified by several authors (Zigmond & Hirsch, 1973; Frei, Baisero & Ochsner, 1974; Keller, Borel, Wilkinson, Hess & Cottier, 1972; Gallin, Clark & Kimball, 1973), has been the method of choice in the *in vitro* assessment of chemotaxis. The method, however, may not be a

perfect assay of spontaneous locomotion of PMNs (Ramsey, 1974). Cutler (1974) was the first to use agarose technique in studies on the chemotaxis of guinea-pig PMNs. In Cutler's method, cells were applied to one agarose well and chemotactic agents to another, and movement of cells toward the attractant was then compared with that toward an appropriate reference substance. Recently, the leucocyte migration agarose test (LMAT) has also been applied to evaluate the migration of human PMNs (Nelson, Quie & Simmons. 1975; John & Sieber, Jr, 1976; Repo, 1977; Repo & Kosunen, 1977).

Although LMAT appears to be well suited for evaluation of spontaneous PMN locomotion, the results of its use in chemotaxis assays are ambiguous. In LMAT, chemotactic agents diffuse toward one side of the cell well and cause deviation of cell migration toward the attractant. This may result from either enhanced random locomotion (chemokinesis) or directional response (chemotaxis) of the cells, or both. If there is chemotactic locomotion, the maximum directional migration should exceed the highest random locomotion. Such a difference between chemotactic and chemokinetic locomotion has been demonstrated by membrane filter techniques (Wilkinson, 1974a; Gallin & Rosenthal, 1974).

In the present study, three different attractants, i.e. casein, bacterial culture filtrate (BCF) and zymosan activated serum (ZAS), were used in LMAT to study the locomotion of normal human peripheral blood leucocytes and to outline the optimal assay conditions for them. It will be shown that each attractant exerted a marked chemokinetic effect on human PMNs. In addition, ZAS also induced an excellent chemotactic response as assayed with LMAT. A comparison with the same cells assayed by a modification of the membrane filter technique using the three attractants was also made. To obtain broad knowledge on cell locomotion *in vitro*, a combination of methods and attractants is needed.

MATERIALS AND METHODS

Cells

Peripheral blood obtained from healthy volunteers 20 to 40 years of age was mixed with preservative-free heparin (Medica Co., Helsinki), 25 iu/ml. Buffy coat cells were separated by the Böyum two-phase method (Böyum, 1974) as described earlier (Repo, 1977). In one experiment, separated PMNs were obtained from buffy coat cells by Ficoll-Isopaque density gradient centrifugation followed by lysis of red blood cells with distilled water (Repo & Kosunen, 1977; Böyum, 1974). The cells were washed in Hanks's balanced salt solution and resuspended in cell medium at an appropriate cell density.

Attractants

Zymosan activated serum (ZAS) was prepared as described earlier (Repo, 1977), and zymosan particles were removed by centrifugation. Heated ZAS was obtained by incubating ZAS for 30 min at 56°. To prepare bacterial culture filtrate (BCF), *E. coli* was grown in Hanks's solution for 24 h at 37°, and the culture fluid was used after sterilization with a 0·22 μm pore size membrane filter (Millipore Corp.). A 2·5% casein solution for LMAT and 0·5% for LFM were prepared by dissolving casein (Hammarsten, E. Merck, Darmstadt) in Hanks's solution by alkali hydrolysis followed by neutralization with HCl. For some LMAT experiments, 8% casein was similarly prepared except that neutralization was achieved by dialysis against Hanks's solution for 72 h at +4°. Inactivated (30 min at 56°) pooled normal human serum (NHS), Hanks's solution, and 2·5% human serum albumin in Hanks's solution (HSA, AB Kabi, Stockholm), were employed as reference substances for serum-derived attractants (ZAS and heated ZAS), BCF and casein, respectively.

Agarose test

The preparation of 1·0% agarose medium (Biomedical Division of Marine Colloids Inc., Rockland, Maine), containing 0·5% HSA, has been described previously (Repo, 1977). Disposable Tissue Culture Dishes 35 × 10 mm and 60 × 15 mm (Falcon Plastics, Oxnard, CA., U.S.A.) were filled with 2·4 ml and 5·0 ml of agarose medium, respectively.

When cell migration toward an attractant (chemotaxis) was studied, sets of three wells in a straight row were cut in agarose. The wells, 3 mm apart, were filled with 5 μl samples of attractant, cell suspension, or appropriate reference substance. When cell migration toward a reference substance (spontaneous locomotion) was determined, the attractant well was filled with the reference substance. If not stated

otherwise, attractant and reference substance were applied twice to their wells (1 h before and simultaneously with the filling of the cell well). After incubation at 37° in a mixture of 2% CO_2 in air, a 3·5% formaldehyde solution was applied to the dishes for 12 to 24 h as a fixative. Agarose gel was then removed, and the procedure described earlier (Repo, 1977) was used to determine the areas and distances of migration toward the attractant and the reference substance.

For experiments in which enhanced random locomotion induced by chemotactic agents (chemokinesis) was studied, an attractant and a reference substance were incorporated in cell media as well as in agarose before gel formation. Single wells at least 10 mm apart were then cut in the gels and filled with cell suspensions. After incubation and fixation as described above, the whole migration area was mapped planimetrically, and the area of the cell well was subtracted. The migration distance was determined with a ruler as follows: two diameters of cell well were subtracted from two perpendicular diameters of migration area, and the difference was divided by four.

Membrane filter technique

The leading front modification (LFM, Zigmond & Hirsch, 1973) of the Boyden chamber technique (Boyden, 1962) was employed according to Wilkinson (1974c), with minor modifications. Buffy coat cells containing $2·0 \times 10^5$ PMNs were placed above a 3 μm pore size membrane filter (Millipore Corp.). Appropriate dilutions of attractants were applied below or on both sides of the filter. After incubation for 55 min as above, the filters were processed for microscopy. Distance of cell front from the starting

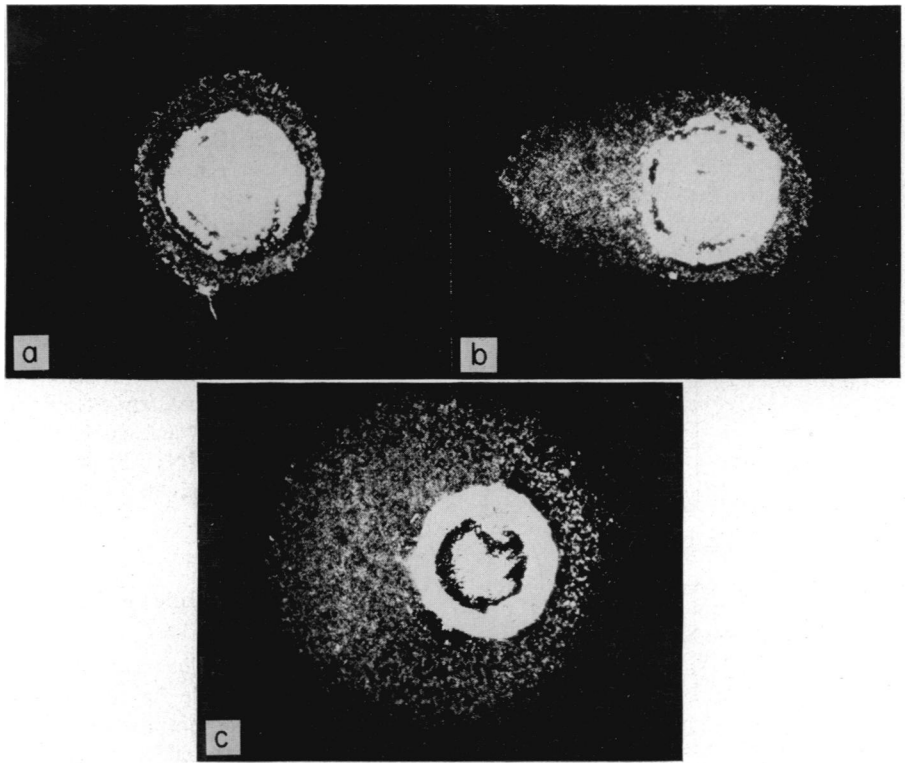

Figure 1. Migration patterns of $2·5 \times 10^5$ separated human PMNs under agarose after 4 h incubation. In each case, the attractant well was situated on the left hand side and the control well on the right hand side of the cell well (seen in white). In (a), there is a reference substance (inactivated NHS) on both sides of the cell well. In (b), migration is seen to be enhanced toward the attractant ZAS only, whereas in (c), it is increased toward both the attractant BCF and the reference substance (Hanks' solution). Magnification × 10.

point was determined as an average of five microscopical fields (magnification ×400) of each of the triplicate filters prepared.

Statistical analysis

Differences between mean migration values of one donor's leucocytes in the presence of attractant and reference substance were evaluated by Student's *t*-test. Comparison between mean migration distances of several donors' leucocytes in the presence of attractant or reference substance, as well as with and without a gradient of attractant were made by Student's *t*-test for matched pairs (Harnett & Murphy, 1975).

RESULTS

Enhanced cell locomotion under agarose toward an attractant (Figs 1 and 2)

Various cell types migrate out of wells cut in agarose, moving predominantly under the agarose layer (Carpenter, Barsales & Ganchan, 1968; Clausen, 1971). Examples of cell migrations under agarose are seen in Fig. 1. The cells used in this case were separated human PMNs that were applied to a central well photographed in the figure. An attractant

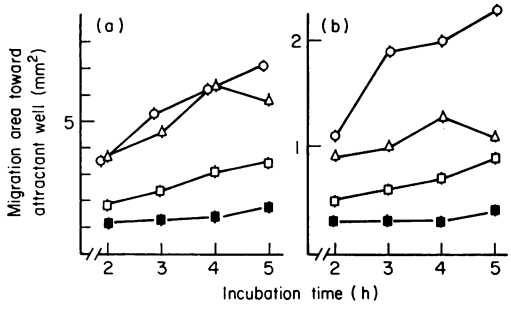

Figure 2. Migration areas (a) and distances (b) under agarose toward attractant wells filled with ZAS (○), BCF (△), 2·5% casein (□), and inactivated NHS (■). The cell wells contained buffy coat cells ($2{\cdot}5 \times 10^4$ PMNs) obtained from one blood donor. Mean ± SE of four migrations per point.

was placed in a similar well 3 mm to the left of the cells, as well as a reference substance to the right of the cell well. In Fig. 1a, however, a reference substance was situated as a control on both sides of the cell well. As can be seen in Fig. 1b, after 4 h incubation, migration of PMNs was directed with a sharp point toward a well containing ZAS as an attractant. A different pattern of cell migration was seen when BCF was applied (Fig. 1c), since it enhanced cell migration not only toward the attractant but also toward the control well.

Cell locomotion under agarose can be determined quantitatively, for instance by measuring the migration areas and distances (Repo, 1977). After the differences in the shapes of migration patterns of separated PMNs in the presence of ZAS and BCF (Fig. 1) had been noticed, a quantitative experiment was performed with human buffy coat cells, using various incubation times. The results in Fig. 2 show that migration areas toward ZAS and BCF were much alike, whereas migration distances toward the former were markedly longer than those toward the latter. Thus the shapes of migration areas are bound to be different, as was also found visually in the previous experiment, using separated PMNs (Fig. 1). The long migration distance of cells in the presence of ZAS has proved to be a constant finding under the assay conditions used and is also true when heated ZAS is used as an attractant (Repo & Kosunen, 1977).

An unexpected finding of the experiment illustrated in Fig. 2 was the rather poor enhancement of migration by casein (2·5%), which is known to be a potent chemotactic substance in other assay systems (Wilkinson, 1974b). Therefore, this experiment was repeated using a shorter distance between the attractant and cell wells (2 mm instead of 3 mm). Even then, migration distance and area were remarkably small compared with those in the presence of ZAS and BCF. Next, an attempt was made to enhance migration toward casein by using an 8% solution. However, PMNs were not found to migrate more, even when this casein preparation was applied hourly up to five times.

Inhibition of cell locomotion under agarose toward an attractant (Fig. 3)

The next experiment, illustrated in Fig. 3, shows that LMAT is also suitable for testing whether attractants, when their concentration is high enough, have inhibitory or reversed effects on cell locomotion. In this case the wells were 2 mm apart, the cell wells were filled with $2{\cdot}5 \times 10^4$ PMNs, and ZAS or BCF was repeatedly added at 1 h intervals into the attractant wells (one application in a 1 h assay, five applications in a 5 h assay). Figure 3a shows that

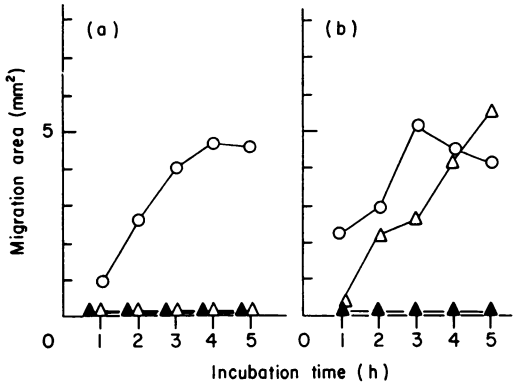

Figure 3. Migration areas of buffy coat cells ($2{\cdot}5 \times 10^4$ PMNs) under agarose toward ZAS (a) and BCF (b), repeatedly applied on an hourly basis. Curve (○) indicates migration areas toward these attractants and curve (△) those toward corresponding reference substances. Increased migration toward ZAS but not toward its reference substance (inactivated NHS) was found as a function of time, whereas migration area toward BCF decreased after 3 h, and that toward the reference substance (Hanks's solution) increased. No migration was detected toward attractant well filled with inactivated NHS (▲). One migration assay per point.

the area of migration toward the repeatedly applied ZAS increased as a function of time, but there was no change in the migration area of the same cells toward the control well filled with the reference substance (inactivated NHS).

A totally different result was obtained in the case of BCF, since the migration area toward hourly applied BCF increased during a 3 h incubation period but decreased thereafter (Fig. 3b). In addition, the corresponding migration areas toward the hourly added reference substance (Hanks's solution) were found to increase especially after 3 h of incubation. The same phenomenon was also found in migration distances. Furthermore, in a microscopical examination of migration areas, PMNs were found to be evenly scattered at 3 h, whereas at 5 h, the cells were found to be densely packed next to the border of the migration area facing the BCF well. The results, repeated using leucocytes of several other donors, suggest that reapplied BCF exerted an inhibitory effect on cell locomotion, and that migration back toward the cell well had occurred after the third hour of incubation. In addition, enhanced cell migration toward the reference substance was seen. These findings led us to study the effect of our attractants on cell locomotion in the absence of a concentration gradient.

Enhanced cell locomotion in an attractant incorporated in agarose and cell medium (Table 1)

Enhanced random locomotion (chemokinesis) induced by chemotactic agents without a concentration gradient has been described using various *in vitro* methods (Wilkinson, 1974a; Keller & Sorkin, 1966; Meier *et al.*, 1955; Gallin & Rosenthal, 1974). We used LMAT and human buffy coat cells. The results, shown in Table 1, indicate that the migration areas of human PMNs under agarose were significantly larger when ZAS, BCF or casein was used than when the corresponding reference substances had been

Table 1. Chemokinetic responses of human PMNs under agarose to ZAS, BCF, and casein

Cell donor	Migration area†					
	ZAS	Reference substance	BCF	Reference substance	Casein	Reference substance
1	$35{\cdot}6 \pm 1{\cdot}4$‡	$28{\cdot}1 \pm 1{\cdot}2$	$35{\cdot}4 \pm 0{\cdot}3$‡	$15{\cdot}9 \pm 1{\cdot}7$	$29{\cdot}2 \pm 0{\cdot}4$‡	$19{\cdot}2 \pm 1{\cdot}0$
2	$37{\cdot}8 \pm 1{\cdot}4$‡	$32{\cdot}6 \pm 0{\cdot}9$	$35{\cdot}4 \pm 0{\cdot}9$‡	$17{\cdot}5 \pm 1{\cdot}0$	$30{\cdot}0 \pm 1{\cdot}1$‡	$19{\cdot}4 \pm 0{\cdot}5$
3	$36{\cdot}4 \pm 4{\cdot}5$§	$28{\cdot}6 \pm 2{\cdot}8$	$34{\cdot}9 \pm 2{\cdot}1$‡	$17{\cdot}0 \pm 0{\cdot}9$	$30{\cdot}6 \pm 0{\cdot}8$‡	$21{\cdot}4 \pm 0{\cdot}9$
4	$32{\cdot}1 \pm 1{\cdot}6$‡	$25{\cdot}9 \pm 0{\cdot}5$	$35{\cdot}1 \pm 0{\cdot}7$‡	$14{\cdot}7 \pm 0{\cdot}6$	$31{\cdot}8 \pm 0{\cdot}7$‡	$17{\cdot}0 \pm 1{\cdot}5$

* Buffy coat cells were obtained from four donors.

† Mean ± SD of six migrations (mm^2) of $2{\cdot}5 \times 10^5$ PMNs after 20 h incubation. Agarose and cell medium contained 10% of either ZAS, BCF, 2·5% casein, or the corresponding reference substances, i.e. inactivated NHS, Hanks's solution, or 2·5% HSA in Hanks's solution, respectively

‡ Significantly different ($P < 0{\cdot}001$) when compared with locomotion in the presence of the reference substance.

§ Significantly different ($P < 0{\cdot}01$) when compared with locomotion in the presence of the reference substance.

incorporated in the agarose gel, i.e. chemokinesis was evident. In addition, it is of interest that migration areas under agarose containing inactivated NHS (reference substance of ZAS) were significantly larger than those in the presence of Hanks's solution or HSA (reference substances of BCF and casein, respectively). This implies that inactivated NHS was chemokinetic although, when it was applied to the attractant well, enhanced migration toward it could not be demonstrated (Figs 1, 2 and 3). Migration rates in the presence of heated ZAS (not shown) were found to be very similar to those in the presence of ZAS.

Directional locomotion and enhanced random locomotion assayed by LMAT and LFM (Tables 2, 3 and 4)

If the cells respond directionally, it should be possible to show that their maximum migration rate toward an attractant is higher than that in the presence of the same attractant without a concentration gradient (Wilkinson, 1974a; Gallin & Rosenthal, 1974). Since it is still questioned whether enhanced cell migration under agarose toward an attractant represents increased random locomotion rather than directional locomotion, LFM and LMAT were used in parallel in the next experiment to test the latter method. Various attractant concentrations and incubation times were used in LMAT to provide optimal migration conditions for the cells.

Table 2 demonstrates the results obtained by LMAT using ZAS as an attractant. Even when diluted 1 : 4 it proved to attract cells significantly both at 4 and 20 h ($P<0.01$). The most effective concentration incorporated in agarose was 12·5% but even 2·5% was enough at 20 h ($P<0.01$). The longest migration distances toward ZAS at 4 h and 20 h (1·9 and 2·2 mm, respectively) were longer than those in ZAS (1·4 and 1·7 mm, respectively),

Table 2. Effect of ZAS on the locomotion of human PMNs

Method*		Migration distance†	
		Incubation time (h)	
LMAT		4	20
Toward inactivated NHS		1·1 ± 0·2	1·3 ± 0·3
Toward ZAS		1·9 ± 0·6‡	2·2 ± 0·8§
Toward ZAS diluted 1 : 2		1·6 ± 0·5	1·9 ± 0·7
Toward ZAS diluted 1 : 4		1·4 ± 0·4	1·6 ± 0·7
Under agarose containing ZAS (v/v)	0·5%	1·2 ± 0·4	1·4 ± 0·6
	2·5%	1·3 ± 0·4	1·6 ± 0·6
	12·5%	1·4 ± 0·2‡	1·7 ± 0·6§
	32·5%	1·2 ± 0·2	1·4 ± 0·3

LFM — Percentage of ZAS:

Above filter	Below filter				
	0	1·5	3·0	6·0	12·0
0	21 ± 4				
1·5		53 ± 6	55 ± 7	54 ± 8	56 ± 9
3·0		52 ± 4	53 ± 5	56 ± 9	60 ± 7
6·0		53 ± 8	54 ± 5	58 ± 7	58 ± 7
12·0		60 ± 4	58 ± 8	59 ± 6	66 ± 8

* In LMAT, the attractant and cell wells were 3 mm apart. Quadruplicate assays of buffy coat cells ($2·5 \times 10^5$ PMNs) were performed. In LFM, migration distances on the right hand side of the underlined figures show locomotion in a positive gradient, and those on the left hand side indicate locomotion in a negative gradient. Underlined figures from upper left to lower right show locomotion in increasing concentration of ZAS in the absence of gradient.

† Mean ± SD of nine donors expressed in mm for LMAT and in μm for LFM.

‡ Significantly different ($P<0.02$) from each other.

§ Significantly different ($P<0.01$) from each other.

Table 3. Effect of BCF on the locomotion of human PMNs

Method*	Migration distance†						
	Undiluted BCF	BCF diluted					Hanks' solution
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
LMAT							
Toward Under agarose containing BCF	1·7 ± 0·2§	ND‡	ND	ND	ND	ND	0·7 ± 0·2
	1·1 ± 0·1¶	1·6 ± 0·1§	1·3 ± 0·1§	1·0 ± 0·1**	1·0 ± 0·1**	0·8 ± 0·1	0·7 ± 0·2
LFM							
Toward Into filter having BCF on both sides	23 ± 4	23 ± 2	24 ± 3	26 ± 2	22 ± 1	22 ± 3	23 ± 4
	18 ± 1§	21 ± 1	22 ± 3	24 ± 2	24 ± 2	24 ± 3	

* In LMAT, the attractant wells were filled thrice (1 h before and simultaneously with the application of cells, as well as after the first hour of incubation). The final dilution of undiluted BCF incorporated in agarose and cell media was 1 : 3. Quadruplicate assays using buffy coat cells ($2·5 \times 10^5$ PMNs) were incubated for 4 h.

† Mean ± SD of seven donors expressed in mm for LMAT and in μm for LFM.

‡ ND = not done.

§ Significantly ($P < 0·001$) different from locomotion in Hanks's solution.

¶ Significantly ($P < 0·01$) different from locomotion in Hanks's solution.

** Significantly ($P < 0·05$) different from locomotion in Hanks's solution.

and thus ZAS can be considered to have exerted a chemotactic as well as a chemokinetic effect on cell migration in LMAT. However, this was not true for the mean migration values in LFM (Table 2) because the longest mean chemotactic migration distance (60 μm) proved to be somewhat shorter than the corresponding chemokinetic migration (66 μm). In addition, when the migration values of individual donors' PMNs were analysed, only three of the nine cell populations summarized in Table 2 showed higher migration distances along a positive gradient of ZAS than in the absence of this gradient.

Having noticed the disagreement between the responses of PMNs under agarose and in membrane filters to ZAS, we next used BCF as an attractant. Since in earlier LMAT experiments it had been found that maximal PMN migration was obtained when BCF was applied to its well three times and that migration toward a well containing BCF diluted in Hanks's solution was always weaker than toward undiluted BCF, only the latter was used in the experiment shown in Table 3. However, when mixed with cells and agarose gel, BCF was found to enhance cell migration even when diluted up to 10^{-4}. The longest migration distance toward BCF (1·7 mm) was not longer than in the presence of BCF (1·6 mm), which implies that deviation of cell migration towards BCF can be ascribed to enhanced random locomotion. As shown in Table 3, our BCF did not attract PMNs into membrane filters at all, but exerted an inhibitory effect on random PMN locomotion when applied undiluted to both sides of the filter.

According to a recent report by Wilkinson (1976), only in the presence of HSA do low molecular weight

Table 4. Effect of casein on the locomotion of human PMNs

Method*	Migration distance†
LMAT	
Toward 2·5% HSA in Hanks's solution	0·6 ± 0·2
Toward casein	1·3 ± 0·1‡
Under agarose containing casein	1·4 ± 0·1‡
LFM	
Toward Hanks's solution	21 ± 3
Toward casein	112 ± 20‡§
Into filter having casein on both sides	82 ± 19‡§

* In LMAT, the wells were 2 mm apart. A 2·5% casein solution was used in the attractant well. In agarose and cell media, the final concentration of casein was 0·5%. Quadruplicate assays using buffy coat cells ($2·5 \times 10^5$ PMNs) were incubated for 4 h. In LFM, a 0·5% casein solution was used.

† Mean ± SD of fourteen donors expressed in mm for LMAT and in μm for LFM.

‡ Significantly ($P < 0·001$) different from locomotion toward reference substance.

§ Significantly ($P < 0·001$) different from each other.

substances such as fatty acids express chemotactic activity. This led us to use LFM to assay PMN migration toward BCF containing HSA. The mean migration distance ± SD of leucocytes of nine donors toward Hanks's solution, Hanks's solution containing 0·2% HSA and undiluted BCF containing 0·2% HSA were 24 ± 4 μm, 33 ± 5 μm and 46 ± 7 μm, respectively. The differences between the last two as well as between the first two values are significant ($P < 0·001$), showing that the cells responded both to BCF in our HSA containing solution and to HSA alone.

Table 4 shows the results obtained by LMAT and by LFM using casein as attractant. In LMAT, cell locomotion was significantly ($P < 0·001$) enhanced both toward casein and in the presence of casein in the agarose gel. There was no significant difference, however, between these two migration distances (1·3 versus 1·4 mm). On the contrary, when determined by LFM, chemotactic migration was significantly longer than the chemokinetic one (112 μm versus 82 μm, $P < 0·001$).

DISCUSSION

Several cell types, including PMNs, have an ability to move about spontaneously. Chemotactic agents are thought to influence this locomotion in at least two different ways. Attractant molecules may stimulate random locomotion (chemokinesis). Secondly, random locomotion may be modified to be appropriately unidirectional (chemotaxis). Evaluation of spontaneous locomotion is required for distinguishing a defect in the intrinsic locomotor capacity of cells from a defect in their chemotactic or chemokinetic responsiveness to an attractant.

Phagocyte migration *in vitro* under an agarose gel is well documented (Cutler, 1974; Nelson *et al.*, 1975; John & Sieber, Jr, 1976; Repo, 1977; Repo & Kosunen, 1977; Carpenter *et al.*, 1968; Clausen, 1971). LMAT seems to be suited for evaluation of the spontaneous locomotion of migrating cells and, as shown in the present study, of chemokinesis and chemotaxis also. To prove that directional migration does exist, paths of a substantial number of individual cells should be followed, which is a cumbersome task. If the maximal migration of a cell population is higher toward an attractant than in an attractant mixed with the culture media, the difference indirectly suggests that the cells respond directionally to the attractant. There is ample evidence for this from LFM experiments (Wilkinson, 1974a; Gallin & Rosenthal, 1974). In LMAT, chemotactic molecules from the attractant well without doubt diffuse into the agarose, but this does not necessarily also indicate the existence of a gradient under agarose, where the PMNs migrate. Diffusion has a role, since migration rates toward preapplied ZAS have been found to be higher than those toward ZAS applied simultaneously with the cells (Repo & Kosunen, 1977). Furthermore, it is not known whether the presence of the cell well modifies the spreading of chemotactic molecules. On the other hand, the presence of a concentration gradient in LFM across a Millipore filter is also difficult to prove.

In the present experiments, two assay systems, LMAT and LFM, were used in parallel to study the response of human PMNs to three attractants differing from each other in many ways. One of the differences was the molecular weight (mol. wt). Chemotactic casein fraction is a large molecule, mol. wt 20–30,000, whereas active factors in *E. coli* culture filtrates have been reported to be small, mol. wt less than 2000 (Wilkinson, 1974b; Schiffman, Showell, Corcoran, Ward, Smith & Becker, 1975). ZAS contains splitting products of activated complement, one of which (C5a) appears to be chemotactic and has an estimated mol. wt of 14,000 (Schiffman *et al.*, 1975). It was found that response of normal human PMNs to these three substances differed from each other in the assays. Casein induced a good chemokinetic migration in LMAT and was the best attractant to demonstrate chemotaxis in LFM. Using ZAS, a chemotactic response, i.e. mean migration distance longer toward rather than in ZAS, was seen in LMAT but not in LFM, except for cells of occasional individual donors. The stimulatory effect of BCF in cell migration in LMAT could be explained by enhanced random locomotion. These findings show that attractants behave differently in various cell locomotion assays, perhaps owing to their different physicochemical properties. The results give credence to the view that responses of cells should be tested using more than one attractant and method, for instance by using ZAS in LMAT or casein in LFM to assay chemotaxis, or casein in LMAT for chemokinesis studies.

The migration distance of PMNs under agarose was found to be markedly longer towards ZAS than towards BCF or casein. Casein induced a migration

pattern similar to but markedly smaller than BCF. These differences in the migration patterns of PMNs under agarose could be explained by dissimilar diffusions of chemotactic molecules from the attractant well, since high mol. wt substances are likely to diffuse more slowly than low mol. wt substances. Another possible explanation for the differently shaped migration patterns of PMNs under agarose can be found in a recent report by Issekutz & Biggar (1977). According to it and our own unpublished observations, incubation of human PMNs in serum-derived chemotaxins does not result in deactivation of cells, insofar as their migration can still be stimulated by bacterial products. When PMNs are incubated in BCF, however, deactivation occurs since after incubation they do not respond to serum derived chemotaxins. This suggests that bacterial products and serum-derived chemotaxins have different modes of action on PMNs. If this is true, the responses of PMNs to the two types of attractants may well result in dissimilar migration patterns of PMNs under agarose.

No inhibitory effect of microbial products on human PMN locomotion was found by Issekutz & Biggar (1977) and in our studies with a 3 mm distance between the wells. However, when a 2 mm distance was used and BCF was applied repeatedly on an hourly basis, 'negative' chemotaxis occurred (Fig. 2). This phenomenon created by bacterial products has also been demonstrated by a membrane filter technique using rabbit PMNs (Schiffman *et al.*, 1975).

Because NHS has been shown to contain chemotactic factors (Stecher, Sorkin & Ryan, 1971), a milieu free of serum components would be advantageous in cell locomotion assays. Unfortunately, the use of a protein such as HSA is necessary in LMAT (Nelson *et al.*, 1975; Repo, 1977), contrary to the membrane filter techniques. Wilkinson (1976) recently demonstrated the requirement of HSA even in LFM when assaying chemotactic activity of low mol. wt substances. Our results show that 0·2% HSA alone enhanced migration of PMNs into Millipore filters, and an additional enhancement was obtained with BCF containing 0·2% HSA, whereas BCF alone proved to be inactive. Low mol. wt substances may require the presence of HSA as a carrier in order to be chemotactic (Wilkinson, 1976), and bacterial products seem to belong to this group of low mol. wt substances.

It appears that a cell capable of migrating directionally in a chemotaxis assay can also enhance its random locomotion in chemokinesis assays, but not necessarily vice versa. Thus, chemokinetic responsiveness may be a prerequisite for chemotaxis, and spontaneous locomotion for chemokinesis. This implies that in a true chemotaxis defect, directional response is impaired, while spontaneous locomotion and chemokinetic responsiveness of the cells are not. To study cell populations of different origin, defects in their spontaneous locomotion and chemokinetic responsiveness should first be taken into account. LMAT, using casein as an attractant, provides an excellent screening test for this purpose. Chemotactic responsiveness should then be determined by various methods, including the use of ZAS in LMAT and casein in LFM, as well as direct observations of the paths of individual cells.

ACKNOWLEDGMENTS

This study was supported by grants from the Sigrid Jusélius Foundation, Helsinki, the Finnish Anti-Tuberculosis Association, Helsinki, and the Emil Aaltonen Foundation, Tampere, Finland. We also thank E. J. Jokinen, M.D., for aid in photography. The skilful technical assistance of Mrs Eine Virolainen and Mrs Maire Laakso is gratefully acknowledged. Part of this article is included in the doctoral dissertation of H. Repo: 'Leukocyte migration agarose test for the assessment of human neutrophil chemotaxis,' University of Helsinki, Helsinki 1976.

REFERENCES

BOYDEN S. (1962) The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. exp. Med.* **115**, 453.

BÖYUM A. (1974) Separation of blood leucocytes, granulocytes and lymphocytes. *Tissue Antigens*, **4**, 269.

CARPENTER R.R., BARSALES P.B. & GANCHAN R.P. (1968) Antigen-induced inhibition of cell migration in agar gel, plasma clot, and liquid media. *J. reticuloendoth. Soc.* **5**, 472.

CLAUSEN J.E. (1971) Tuberculin-induced migration inhibition of human peripheral leucocytes in agarose medium. *Acta Allerg.* (*Kbh.*), **26**, 56.

CUTLER J.E. (1974) A simple *in vitro* method for studies on chemotaxis. *Proc. Soc. exp. Biol. Med.* (*N.Y.*), **147**, 471.

FREI P.C., BAISERO M.H. & OCHSNER M. (1974) Chemotaxis of human polymorphonuclears *in vitro*. II. Technical study. *J. immunol. Meth.* **5**, 375.

GALLIN J.I., CLARK R.A. & KIMBALL H.R. (1973) Granulocyte chemotaxis: An improved *in vitro* assay employing ^{51}Cr-labeled granulocytes. *J. Immunol.* **110,** 233.

GALLIN J.I. & ROSENTHAL A.S. (1974) The regulatory role of divalent cations in human granulocyte chemotaxis. Evidence for an association between calcium exchanges and microtubule assembly. *J. Cell. Biol.* **62,** 594.

HARNETT D.L. & MURPHY J.L. (1975) *Introductory Statistical Analysis*, p. 364. Addison-Wesley, Reading, Mass., U.S.A.

ISSEKUTZ A.C. & BIGGAR W.D. (1977) Influence of serum-derived chemotactic factors and bacterial products on human neutrophil chemotaxis. *Infect. Immun.* **15,** 212.

JOHN T.J. & SIEBER O.F. JR (1976) Chemotactic migration of neutrophils under agarose. *Life Sci.* **18,** 177.

KELLER H.U. & SORKIN E. (1966) Studies on chemotaxis. IV. The influence of serum factors on granulocyte locomotion. *Immunology*, **10,** 409.

KELLER H.U., BOREL J.F., WILKINSON P.C., HESS M.W. & COTTIER H. (1972) Re-assessment of Boyden's technique for measuring chemotaxis. *J. Immunol. Meth.* **1,** 165.

KELLER H.U., WILKINSON P.C., ABERCROMBIE M., BECKER E.L., HIRSCH J.G., MILLER M.E., SCOTTRAMSEY W. & ZIGMOND S.H. (1977) A proposal for the definition of terms related to locomotion of leucocytes and other cells. *Clin. exp. Immunol.* **27,** 377.

MCCUTCHEON M. (1946) Chemotaxis in leukocytes. *Physiol. Rev.* **26,** 319.

MEIER R., DESAULLES P.A. & SCHÄR B. (1955) Verschiedenartiger Wirkungstypus bakterieller 'entzündungserregender' Substanzen und anderer 'Reizstoffe'. *Arch. exp. Path. Pharmak.* **224,** 104.

NELSON R.D., QUIE P.G. & SIMMONS R.L. (1975) Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J. Immunol.* **115,** 1650.

RAMSEY W.S. (1974) Leucocyte locomotion and chemotaxis. *Antibiotics Chemother.* **19,** 179.

REPO H. (1977) Leukocyte migration agarose test for the assessment of human neutrophil chemotaxis. I. Effects of environmental factors on neutrophil migration under agarose. *Scand. J. Immunol.* **6,** 203.

REPO H. & KOSUNEN T.U. (1977) Leukocyte migration agarose test for the assessment of human neutrophil chemotaxis. II. Variables in the attraction assay. *Scand. J. Immunol.* **6,** 211.

SCHIFFMAN E., SHOWELL H.V., CORCORAN B.A., WARD P.A., SMITH E. & BECKER E.L. (1975) The isolation and partial characterization of neutrophil chemotactic factors from *Escherichia coli*. *J. Immunol.* **114,** 1831.

STECHER V.J., SORKIN E. & RYAN G.B. (1971) Relation between blood coagulation and chemotaxis of leucocytes. *Nature* (*New Biol.*), **233,** 95.

WILKINSON P.C. (1974) *Chemotaxis and Inflammation*, p. 36(a), p. 80(b), p. 168(c). Churchill Livingstone, Edinburgh.

WILKINSON P.C. (1976) A requirement for albumin as carrier for low molecular weight leukocyte chemotactic factors. *Exp. Cell Res.* **103,** 415.

ZIGMOND S.H. & HIRSCH J.G. (1973) Leukocyte locomotion and chemotaxis. New methods for evaluation and demonstration of a cell-derived chemotactic factor. *J. exp. Med.* **137,** 387.