

CHEMOTAXIS BY MOUSE MACROPHAGE CELL LINES

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Five mouse macrophage cell lines were tested for chemotaxis and phagocytosis. All 5 cell lines ingested sheep red cells coated with rabbit IgG anti-Forsman antibody and exhibited chemotaxis to endotoxin-activated mouse serum (EAMS) and lymphocyte-derived chemotactic factor. Two cell lines were tested for chemotaxis to f-Met-Leu-Phe and neither responded. Four of the cell lines (RAW264, RAW309CR, PU5-1R, and WR19M.1) exhibited chemotaxis to C5a. These cell lines displayed a 1- to 2-hr lag before migrating toward EAMS, and chemotaxis was dependent upon cell density. When fewer than 10^3 cells were present per mm^2 of filter surface, less than 10% of the cells migrated; however, at a density of 5×10^3 cells/ mm^2 50 to 70% of the cells migrated. WEHI-3 differed from the other cell lines in that there was no chemotaxis to C5a, migration to EAMS did not have a detectable lag, and there was no cell density dependence for chemotaxis. Comparison of these chemotactic properties with those reported in the literature for mouse macrophages and monocytes suggests that RAW264, RAW309CR, PU5-1R, and WR19M.1 have properties that are similar to those of mouse resident macrophages, whereas WEHI-3 may have some of the properties of mouse monocytes.

Mouse macrophages accumulate at inflammatory sites where they may function as immunoregulatory cells or as effector cells with phagocytic and cytolytic activities. This accumulation could occur either by chemotaxis or by trapping of macrophages that randomly wander through the site. The contribution of each mechanism is not known. However, the finding that macrophages exhibit chemotaxis to factors that are generated at inflammatory sites suggests a role for chemotaxis. Chemotactic factors for mouse macrophages include a product of complement activation (C5a) (1, 2), a product of activated lymphocytes (LDCF)¹ (2), and products from bacteria (3).

To study the biologic activities associated with a population of macrophages, these cells are usually separated from other cell types. Collection and separation of the macrophages may require 2 hr or more. In addition, some assessment of contaminating cell types is needed. Removal of nonmacrophage cells may alter the properties of the macrophages. The differentiation state of the macrophage may change due to the time required for separation of the cells and measurement of the activity, to the treatment used in the cell separation, or to the removal of interacting cells. One approach to minimize these problems is the use of macrophage

cell lines.

Several cell lines have been isolated that have properties that are characteristic of macrophages. For example, these cell lines are capable of phagocytosis of latex and antibody-coated sheep red blood cells (SRBC) (4-6). Additional properties of these cell lines have been recently summarized by Morahan and Walker (7). These cell lines have been used to enhance our understanding of the properties of macrophages. For example, macrophage cell lines have been used to study a surface antigen associated with tumoricidal macrophages (8), to study Fc receptors (9-14), to isolate nonphagocytic mutants (15), and to produce soluble mediators (16, 17). In this paper we show that 4 of the cell lines have the chemotactic properties of macrophages and that 1 cell line has chemotactic properties that are more like a monocyte. These findings indicate that cell lines may be good models for macrophage and monocyte chemotaxis and a convenient source of cells for biochemical studies of chemotaxis.

MATERIALS AND METHODS

Cells. Macrophage cell lines were obtained from the Cell Distribution Center, Salk Institute, San Diego, CA, and cultured in modified Eagle's medium (MEM) containing 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (MEM-10%FCS). During culture both adherent and nonadherent cells were observed. For subculture nonadherent cells were discarded, adherent cells were scraped with a rubber policeman into fresh medium, and one-tenth of the cells was used to inoculate new flasks. Except for RAW309CR, the cell lines were subcultured twice a week; RAW309CR grew more slowly and was subcultured once a week. None of the cell lines were contaminated with mycoplasma when tested by Microbiological Associates (Bethesda, MD).

To assay for chemotaxis or phagocytosis both adherent and nonadherent cells were centrifuged at $150 \times G$ for 10 min. The cell pellet was resuspended in MEM-10%FCS and the number of viable cells was determined by trypan blue exclusion.

Soft agar cloning. Macrophage cell lines were cultured continuously for approximately 1 yr. Single-cell suspensions of actively growing cells were suspended in MEM-10%FCS containing 0.3% Bacto-agar (Difco Laboratories, Inc., Detroit, MI) at 40°C and 5 ml containing either 90, 30, or 10 cells were poured onto a solid 0.7% Bactoagar-MEM-10%FCS layer of 4 ml in 60-mm Petri dishes (18). After 13 days, single colonies were removed with a pipet and grown in 16-mm wells. When confluent, the cells were transferred to a 75 cm^2 T-flask. Clones were tested for chemotaxis and phagocytosis within the first 5 passages. The cloning efficiency for RAW264, PU5-1R, and WEHI-3 was 30 to 40%, the cloning efficiency for WR19M.1 was 3%, and no clones were found for RAW309CR.

Chemotaxis assay. Chemotaxis was assayed in a 48-well multiwell chamber as described previously (19). The bottom wells were filled with 25 μl of RPMI 1640 or attractant solution diluted in RPMI 1640 (Grand Island Biological Co., Grand Island, NY). A filter sheet (Nucleopore, 5- μm hole size, Pleasanton, CA) was placed on the bottom plate. A silicon gasket and the top plate with 48 holes were then mounted, forming the top wells. The cells were added in a volume of 40 μl . After a 4-hr incubation the chamber was disassembled, the filter sheet was removed, and nonmigrated cells were wiped off the top side. The filter was then fixed in methanol for 1 min and stained in Diff-Quick (Harleco, Gibbstown, NJ). The cells on the bottom of the filter were counted with an Optomax Image Analyzer (Optomax Inc., Hollis, NH). Two randomly chosen microscope fields were counted, which together represented 13% of the total area (8 mm^2) available for chemotaxis. All assays were performed in duplicate. The standard error of the mean for duplicates was less than 20% when more than 100 cells were counted.

Endotoxin-activated mouse serum (EAMS), partially purified C5a, LDCF, f-Met-Leu-Phe and dipeptides were diluted in RPMI 1640 without FCS and tested as attractants. f-Met-Leu-Phe and dipeptides were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions (10^{-3} M) were prepared in ethanol. EAMS and Con A-induced LDCF from spleen cells were

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¹ Abbreviations used in this paper: f-Met-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; f-Met-Ala, *N*-formyl-L-methionyl-L-alanine; f-Met-Phe, *N*-formyl-L-methionyl-L-phenylalanine; f-Met-Val, *N*-formyl-L-methionyl-L-valine; LDCF, lymphocyte-derived chemotactic factor; MEM, modified Eagle's medium; FCS, heat-inactivated fetal calf serum; EAMS, endotoxin-activated mouse serum; Gey's, Gey's balanced salt solution; PBS, Dulbecco's phosphate-buffered saline without calcium and magnesium.

TABLE I
Chemotaxis by macrophage cell lines^a

Expt. No.	RAW264		RAV309CR		PU5-1R		WR19M.1		WEHI-3	
	Cells per well	Cells migrated ^b	Cells per well	Cells migrated						
11-12	2 × 10 ⁴	6300 (25)	2 × 10 ⁴	5850 (23)	2 × 10 ⁴	850 (3)	2 × 10 ⁴	8250 (33)	2 × 10 ⁴	700 (3)
11-15	1 × 10 ⁴	2400 (24)	1 × 10 ⁴	2050 (21)	1 × 10 ⁵	300 (0)	5 × 10 ³	400 (8)	1 × 10 ⁵	2100 (2)
12-20	1 × 10 ⁴	50 (0)	1 × 10 ⁴	1100 (11)	1 × 10 ⁵	100 (0)	1 × 10 ⁴	1550 (15)	1 × 10 ⁵	3900 (4)
1-15					1 × 10 ⁵	5600 (6)	1 × 10 ⁵	2300 (2)	1 × 10 ⁵	3900 (4)
1-24	1 × 10 ⁵	23600 (24)							1 × 10 ⁵	6100 (6)
1-31	4 × 10 ⁴	9800 (24)	2 × 10 ⁴	4200 (21)	4 × 10 ⁴	4900 (12)	2 × 10 ⁴	9500 (48)		
2-5	3 × 10 ⁴	6800 (23)								
2-14	2 × 10 ⁴	5300 (27)	2 × 10 ⁴	1200 (6)	5 × 10 ⁴	1550 (3)	1 × 10 ⁵	20800 (21)		
2-21					5 × 10 ⁴	500 (1)				
2-24					1 × 10 ⁵	3550 (4)				
3-4	1 × 10 ⁵	42500 (42)			1 × 10 ⁵	38800 (39)	1 × 10 ⁵	41900 (42)		

^a The attractant was a 1:100 dilution of endotoxin-activated mouse serum (EAMS). In the absence of EAMS less than 1% of the cells migrated.

^b When 20,000 to 40,000 cells have migrated, the bottom of the filter is completely covered. The value in parentheses is the per cent of viable input cells that migrated. Before the cells spread in a culture flask, they have a diameter of approximately 12 μm or more than twice the diameter of the holes in the filter separating the cells and attractant.

prepared as described (2). Mouse C5a was prepared from serum generated from anticoagulant-free mouse plasma as follows. Blood from the tail vein of a mouse warmed under a heat lamp was collected into an iced Fisherfuge polycarbonate centrifuge tube, which was then immediately centrifuged for 30 sec. The supernatant plasma was transferred to a glass tube for clotting. This procedure was repeated with a sufficient number of mice to generate 12 ml of serum, 9 of which were mixed with 27 mg of LPS (*S. typhosa* 0901, Product No. 3124-25, Difco Laboratories Inc.) and incubated for 90 min at 37°C followed by 30 min at 56°C. The activated serum was centrifuged for 15 min at 15,000 × G and stored frozen in aliquots. Activated (8 ml) and control sera were chromatographed on Sephadex G-100. A well defined peak of chemotactic activity was eluted from the activated serum column that corresponded to a m.w. of 14,000. The volume of the pooled active fractions was 36 ml. No significant activity was detected in the column eluate when control serum heated at 56°C was chromatographed.

Phagocytosis assay. Phagocytosis was measured by uptake of ⁵¹Cr-labeled, rabbit anti-Forsman IgG-coated SRBC. The red cells were prepared by incubation of 10⁹ red cells with 25 μCi of sodium chromate and 10 μl of an anti-Forsman IgG preparation. After 1 hr the cells were washed twice and suspended in 5 ml of RPMI 1640. Phagocytic cells (1 × 10⁵ to 2 × 10⁵ in 100 μl) were pipetted into V-shaped wells of a flexible microtiter plate (Dynatech Lab. Inc., Alexandria, VA). IgG-coated red cells (10⁷ in 50 μl) were added and the plate was incubated at 37°C for 2 hr. The plates were then centrifuged for 4 min at 800 × G and the supernatant was removed. Nonendocytosed cells were lysed by treatment for 3 min with ammonium chloride lysing buffer. The cells were again pelleted and washed twice with RPMI 1640. The plates were sealed with Microtiter Plate Sealers and the wells were cut out and counted in a Packard Gamma Counter. All assays were done in triplicate. The standard error of the mean for triplicates was less than 7%. In the absence of phagocytic cells, 0.2% of the radioactivity remained in the wells.

RESULTS

Chemotaxis and phagocytosis of macrophage cell lines. Five different macrophage cell lines exhibited chemotaxis to a 1/100 dilution of EAMS. A summary of the chemotactic responses for the first 15 experiments is presented in Table I. The number of cells added varied from 5 × 10³ to 1 × 10⁵ per well and the time of chemotaxis was 4 hr. When 20,000 to 40,000 cells had migrated, the bottom of the filter was completely covered with cells, representing maximum migration. Very few cells dropped off the lower filter surface into the bottom chamber. In addition to exhibiting chemotaxis to EAMS, all of the cell lines exhibited a weak and variable chemotactic response to a 1:2 dilution of the culture fluid from Con A-activated mouse splenocytes (LDCF) (data not shown). PU5-1R² gave the best response to this attractant. Chemotaxis of 2 other macrophage cell lines, P388D1 and J774.1, has been reported (20). P388D1 did not exhibit chemotaxis to EAMS or LDCF. J774.1 exhibited good chemotaxis to EAMS and very poor chemotaxis to LDCF. In this respect, J774.1 resembles the 5 cell lines in this study.

The chemotactic response to EAMS on different days was vari-

able (Table I). WR19M.1 was the most variable; between 2 and 42% of the cells migrated on different days. On the other hand, the percentage of RAW264 that migrated was usually within a narrow range (23 to 27%). Two exceptions are shown in Table I where the percentage of RAW264 that responded was unusually high in one instance and not detectable in the other. Large variations of unusually high or low chemotaxis occurred several times over a period of approximately 1 yr at unpredictable intervals. The changes in chemotactic response were apparently a property of the cells since sister cultures tested on different days also exhibited the abnormal chemotaxis (data not shown). After growing for a few passages the cells exhibited the usual chemotactic response. The reasons for these variations are not known, but 2 factors do not appear to influence the percentage of cells that migrate. First, dead cells do not interfere with chemotaxis. In 2 different experiments the viability of RAW264 determined by trypan blue exclusion was 42 and 97%; however, the same fraction of cells (24%) migrated in both experiments. Second, the time in continuous culture does not influence the percentage of cells that migrate. The chemotactic response of cells cultured for approximately 1 yr (Expts. 11-12 to 12-20) were similar to those cultured for 1 to 3 mo (Expts. 1-31 to 3-4). When all of the data are considered, RAW264, RAW309CR, and WR19M.1 exhibited the strongest chemotaxis to EAMS; PU5-1R and WEHI-3 exhibited the weakest chemotaxis.

The macrophage cell lines were also phagocytic for SRBC coated with rabbit IgG anti-Forsman antibody (Table II). Phagocytosis by WEHI-3 was poor when compared to the other cell lines. No phagocytosis occurred if the red cells were not coated with antibody, indicating that the phagocytic response is probably mediated by the Fc receptor. When the data in Tables I and II are compared, it can be seen that a cell line that exhibits poor chemotaxis does not necessarily have a poor phagocytic response (eg., PU5-1R). Antibody-mediated phagocytosis by the cell lines has been previously reported (5, 6).

Effect of chemotactic medium. Studies of macrophage chemotaxis have employed different media and in some instances the media in top and bottom compartments have not been identical (2, 21). In addition, it has been found that mouse resident peritoneal macrophages do not respond well to EAMS when MEM is present in the lower compartment of a modified Boyden chamber (E. J. Leonard, unpublished observation). For these reasons the effect of different media with and without protein in the top and bottom compartments of a multiwell chemotaxis apparatus was studied for RAW264. In the experiment shown in Table III, the chemotactic responses varied 30-fold and they were grouped into good, intermediate, and poor responses. In a 2nd experiment, very similar results were obtained and the responses could be grouped into the same categories. Intermediate responses were found when the same medium without protein was used in the top and bottom compartments whether it was Gey's, RPMI 1640, or MEM. The chemotactic response was usually increased by including FCS or BSA in the top compartment only and decreased by including FCS or BSA in the bottom compartment only. It is interesting that BSA

² PU5-1R is the same as PU5-1.8 (P. Ralph, personal communication). PU5-1R is used in this paper since it was the designation given by the Cell Distribution Center, Salk Institute, from which the cell line was obtained.

TABLE II
Phagocytosis by macrophage cell lines

Expt. No.	Ingested SRBC per Cell*				
	RAW264	RAW309CR	PU5-1R	WR19M.1	WEHI-3
12-20	1.4	4.2	0.4	1.1	1.2
1-15			1.7	3.2	0.1
1-24	3.4				0.0
1-24a	2.9				
1-31	3.4	6.2	2.6	5.8	
2-7	4.1	4.1	3.7	2.6	
2-14	2.2	2.1	3.1	2.0	

* The uptake of rabbit anti-Forsman IgG-coated sheep erythrocytes, labeled with ⁵¹Cr, was determined at a ratio of 40 SRBC per cultured cell. In the absence of anti-Forsman antibody, there were 0.00 SRBC/cell.

TABLE III
The chemotactic response of RAW264 cells suspended in different media to EAMS diluted in different media*

Chemotactic Response to EAMS (Cells/Attractant)		
Good (>10,400 migrated cells)	Intermediate (5600 to 10400 migrated cells)	Poor (<700 migrated cells)
Gey's/RPMI 1640	Gey's/Gey's	Gey's/MEM Gey's/Gey's-2%BSA Gey's/RPMI 1640-10%FCS Gey's/MEM-10%FCS
Gey's-2%BSA/RPMI 1640 Gey's-2%BSA/MEM Gey's-2%BSA/RPMI 1640-10%FCS Gey's-2%BSA/MEM-10%FCS	Gey's-2%BSA/Gey's-2%BSA Gey's-2%BSA/Gey's	
	RPMI 1640/Gey's	RPMI 1640/RPMI 1640-10%FCS
	RPMI 1640/RPMI 1640	RPMI 1640/MEM-10%FCS
	RPMI 1640/MEM RPMI 1640/Gey's-2%BSA	
RPMI 1640-10%FCS/Gey's RPMI 1640-10%FCS/RPMI 1640 RPMI 1640-10%FCS/Gey's-2%BSA	RPMI 1640-10%FCS/MEM RPMI 1640-10%FCS/RPMI 1640-10%FCS RPMI 1640-10%FCS/MEM-10%FCS	
MEM/Gey's	MEM/RPMI 1640 MEM/MEM MEM/Gey's-2%BSA	MEM/RPMI 1640-10%FCS MEM/MEM-10%FCS
MEM-10%FCS/Gey's	MEM-10%FCS/Gey's-2%BSA	MEM-10%FCS/RPMI 1640-10%FCS
MEM-10%FCS/RPMI 1640	MEM-10%FCS/MEM-10%FCS	
MEM-10%FCS/MEM		

* The attractant was a 1:100 dilution of EAMS and there were 50,000 cells per well. Harvested cells were pelleted and the pellet was washed once in Gey's-2% BSA. The cell pellet was resuspended in Gey's-2%BSA and divided into aliquots. Each aliquot of cells was pelleted and resuspended in the indicated medium. The medium used to resuspend the cells is separated by a slash from the medium used to dilute the attractant. Thus, cells resuspended in MEM-10% FCS and tested for chemotaxis to EAMS diluted in RPMI 1640 is designated MEM-10% FCS/RPMI 1640.

or FCS is not required since it has been found that mouse resident macrophages require a 100,000 m.w. serum protein for chemotaxis (22). Chemotaxis by mouse BCG-elicited peritoneal macrophages is stimulated by a different protein fraction of serum (23).

Time course of chemotaxis. Chemotaxis by the macrophage cell lines to 2 dilutions of EAMS at various times is shown in Figure 1. Migration by the cells was nearly complete in 4 hr; at longer times only a slow increase in the number of migrating cells occurred. At longer times the migrated cells occasionally formed clumps on the

bottom of the filters, which made quantitation difficult. In addition, a significant fraction of the cells may also divide and dividing cells are generally less adherent. An increase in the number of cells through division would make it appear that more than 100% of the cells migrated. Thus, in Figure 1 it was calculated that approximately 100% of the WR19M.1 cells migrated in 7 hr. In this experiment, the WR19M.1 cells may have been exceptionally motile as suggested by the unusually high migration in the absence of attractant. However, it should be noted that the cell number was doubled for WR19M.1 and WEHI-3 when migration was measured in the absence of EAMS. On another occasion, 140% of RAW309CR appeared to migrate.

Four of the cell lines (RAW264, RAW309CR, PU5-1R, and WR19M.1) showed a lag of 1 to 2 hr before they began to migrate; migration was nearly complete in an additional 2 to 3 hr. This suggests that certain changes occur in the cells during incubation before they are capable of responding to the attractant. WEHI-3 did not show an obvious lag and for this cell line migration was nearly complete in 2 hr. The time course of chemotaxis for all 5 cell lines was also measured with twice the number of cells, and the lag time and the time required for completion of migration were the same.

Dependence of chemotaxis on attractant concentration. Optimal chemotaxis to EAMS by the cell lines was usually obtained near a 1/100 dilution (Fig. 2). However, the exact dilution that resulted in optimal chemotaxis, the magnitude of the response (Table I), and the dilution range over which a response was obtained varied between experiments. For example, in other experiments the response of RAW309CR to EAMS occurred over a narrower concentration range similar to that for RAW264 shown in Figure 2.

Although it is possible that activated serum contains many at-

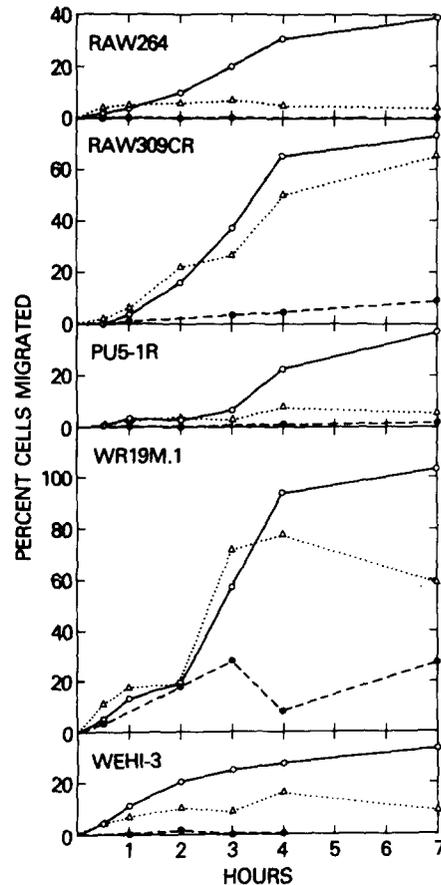


Figure 1. Time course of chemotaxis. Cells were added to the upper wells of multiwell chambers and the filters removed at the indicated times. The lower wells contained RPMI 1640 (●-●), 1:800 dilution of EAMS in RPMI 1640 (Δ-Δ), or 1:100 dilution of EAMS in RPMI 1640 (○-○). In all but 2 cases the wells contained 50,000 cells. For the response of WR19M.1 and WEHI-3 to RPMI 1640, the wells contained 100,000 cells.

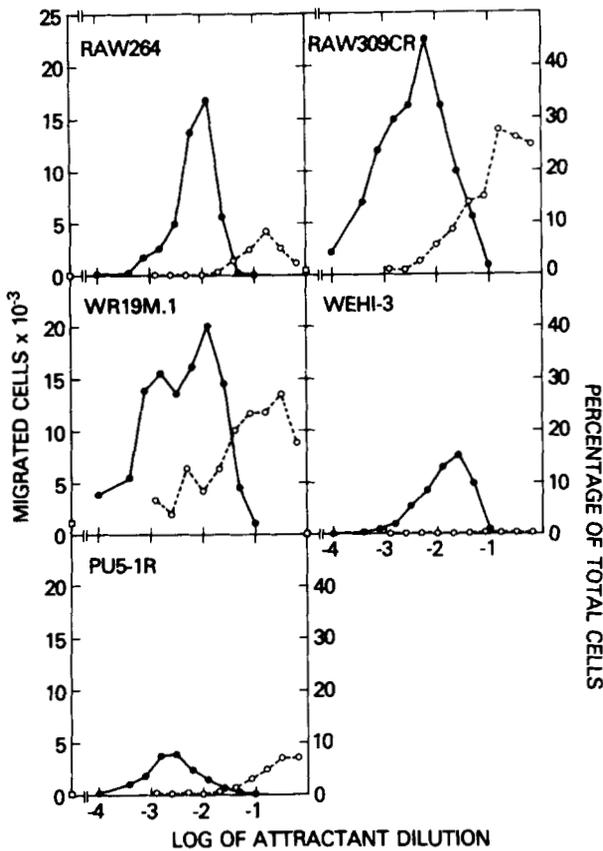


Figure 2. The chemotactic response of macrophage cell lines to various dilutions of EAMS (●—●) and C5a (○---○). Each well contained 50,000 cells. □, The response to medium.

tractants, the major attractant for mouse peritoneal macrophages elicited with proteose peptone is C5a (1). Therefore, the chemotactic response of the cell lines was determined for mouse serum obtained from cell-free plasma that was activated with endotoxin and chromatographed on Sephadex G-100. A single peak of chemotactic activity for mouse resident macrophages was found that corresponded to a m.w. of 14,000, similar to the m.w. of C5a. The most active fractions were pooled and used as an attractant for the mouse macrophage cell lines. Four of the cell lines exhibited chemotaxis to purified EAMS (C5a, Fig. 2). The chemotactic response of RAW264, RAW309CR, PU5-1R, and WR19M.1 to C5a was optimal at a 1:3 dilution. For RAW264, RAW309CR, and WR19M.1 the optimal response to C5a was lower than the optimal response to EAMS. This suggests that these cell lines may respond to other attractants or to factors that augment the C5a response. RAW264 also responded to a purified preparation of human C5a.

WEHI-3 did not respond to mouse C5a (Fig. 2). However, it did respond to EAMS. This demonstrates that EAMS contains at least 1 attractant with a m.w. substantially different from that of C5a. An attractant for macrophages elicited with proteose peptone that has a m.w. greater than 68,000 on Sephadex G-100 has been reported in mouse serum (1).

Since macrophages and neutrophils from other species exhibit chemotaxis to *N*-formylmethionyl peptides (24, 25), RAW309CR and PU5-1R were tested for chemotaxis to f-Met-Leu-Phe and some related dipeptides. Neither of the cell lines exhibited a chemotactic response to 10^{-8} , 10^{-7} , 10^{-6} , or 10^{-5} M f-Met-Leu-Phe, f-Met-Phe, f-Met-Val or f-Met-Ala. In these experiments both cell lines gave the expected chemotactic response to several concentrations of EAMS demonstrating that the cells were capable of chemotaxis.

Dependence of chemotaxis on cell density. As an increasing number of cells are added to the top compartment of a chemotaxis chamber, the filter should become saturated. To test this the number of cells in the top compartment was varied and the chemotactic response to EAMS was measured (Fig. 3). RAW309CR,

PU5-1R, and RAW264 gave similar results. At low cell densities chemotaxis was poor. When the cell density was greater than 8×10^3 cells/well (10^3 cells/mm²), the fraction of the input cells that migrated increased sharply. In this region of the curve 50 to 70% of the input cells migrated. Apparent saturation of the filter surface occurred at densities above 8×10^4 cells/well (10^4 cells/mm²) in the upper chamber. The cell density dependence of chemotaxis by WR19M.1 was not as critical as for RAW309CR, PU5-1R and RAW264. No requirement for a certain density of cells was observed for WEHI-3 (clone 1). These data suggest that cell interactions may increase the chemotactic response for all the cell lines except WEHI-3.

Chemotaxis and phagocytosis of cell line clones. One explanation for the failure of a fraction of the cells to migrate toward an attractant is that the cells are a mixture of stable subpopulations of chemotactic and nonchemotactic cells. To test if the cell lines contained nonchemotactic subpopulations, cell lines that had been in culture for approximately 1 yr were cloned. The cells were cloned in soft agar since the cultured cells are both adherent and nonadherent. Clones were obtained from all cell lines except RAW309CR. The cloned cell lines were tested within a few passages to minimize any spontaneous reversion of the chemotactic properties of the cells. Since the properties of each cloned cell line are representative of 1 original cell in the uncloned population, the magnitude of the response is not as important as whether the cloned cell lines are chemotactic. All of the cloned cell lines were

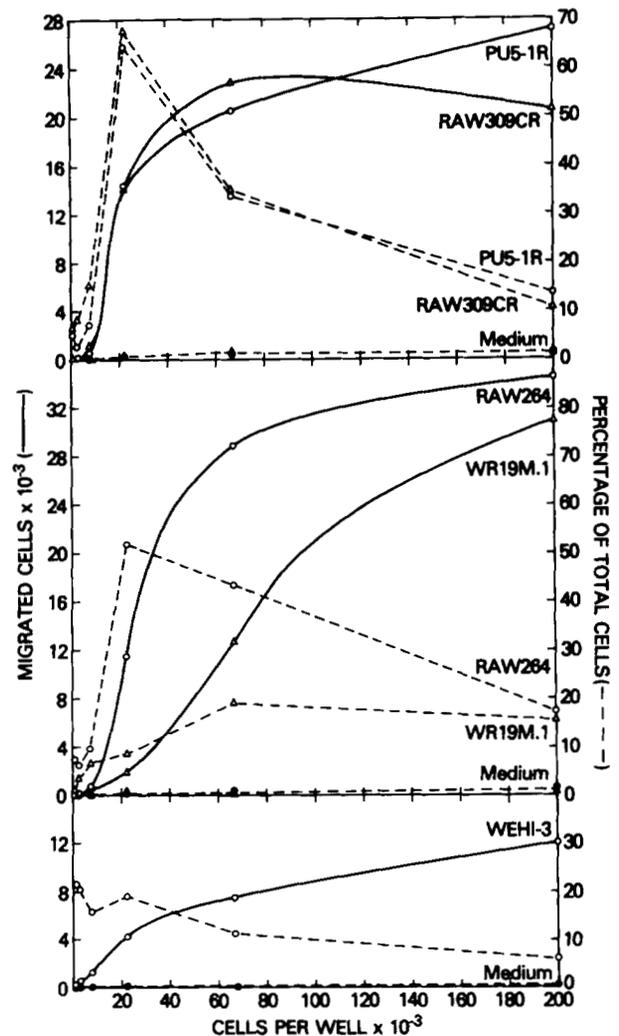


Figure 3. The chemotactic response of macrophage cell lines at various cell densities. The number of cells added to each well is shown on the abscissa. Migration measured to a 1:100 dilution of EAMS is expressed both as the total number of cells that migrated and as the percentage of added cells. The number of cells that migrated to medium is indicated by the closed symbols.

chemotactic and phagocytic (Table IV) and their responses were within the variability of the assays determined for the parental cell lines (compare Table I). When the cloned cell lines were tested a 2nd time the activities were similar in magnitude, but the relative ranking of the cells by activity changed, suggesting that the variations among the clones were random. One clone, WEHI-3 (clone 1) was cultured for 20 passages and tested for chemotaxis to EAMS in 9 separate experiments. The percentage of cells that migrated varied from 3 to 12%. Since all of the clones were chemotactic and phagocytic, these data suggest that nonchemotactic or nonphagocytic cells in the parental population have the potential to exhibit chemotaxis and phagocytosis.

Selection for chemotactic cells. Although all of the clones from the cell lines were chemotactic and phagocytic, the clones were derived from only a few cells in the culture. To obtain additional evidence that a stable subpopulation did not exist, cultures were initiated from the migrated cells. Because of their larger size, standard blindwell chemotaxis chambers (Neuroprobe Corp., Bethesda, MD) were used in these experiments. After cells had migrated toward EAMS, the tops of the filters were wiped to remove nonmigrated cells. Some of the filters were counted to determine the number of cells that migrated; other filters with migrated cells were grown in culture. When the cells had grown to a sufficient number, the selection was repeated. The results of these experiments are presented in Table V. RAW264, PU5-1R and WEHI-3 appeared to be enriched by the first selection since approximately twice as many cells migrated during the 2nd selection. Although the chemotactic response was poor, no increase in the number of migrating cells was found for WR19M.1. The percentage of migrating RAW264 cells continued to increase with each selection until the 4th. Then for unknown reasons the cells did not migrate well. Although it appears that the selection procedure was enriching for migrating cells, it is possible that the apparent increase in migrated cells was only a reflection of the variability of chemotaxis (Table I). If some selection for migrating cells was obtained, it was not as large as expected. The wiping procedure was very efficient and very few cells were observed on the top of the filters. In addition, on those occasions when migrated cells were not scraped from the filter during culture, clones appeared only on the bottom of the filter. The data from the cloning and selection experiments suggest that few stable nonchemotactic cells exist and that nearly every cell is capable of migration. Whether or not a cell will migrate depends upon its physiologic state.

TABLE IV
Chemotaxis and phagocytosis by cell line clones^a

Cell Line	Clone	Chemotaxis			Phagocytosis	
		1:100 EAMS	1:2 LDCF	Medium		
		% migrated			SRBC/cell	
RAW264		33.6	0.1	0.3	2.9	
	1	1.5	0.2	0.3	0.6	
	2	6.4	0.1	0.0	2.6	
	3	11.7	0.1	0.0	2.6	
	4	3.1	0.3	0.1	0.7	
PU5-1R		5.8	0.6	0.3	1.7	
	1	1.7	2.0	0.1	0.4	
	2	6.0	2.1	0.2	0.6	
	WR19M.1		23.8	9.8	0.6	3.2
		1	8.8	3.8	0.7	2.3
2		1.7	0.2	0.6	0.9	
WEHI-3	1	9.4	0.6	0.1	3.4	
	2	12.7	1.0	0.1	3.3	
	3	5.0	0.3	0.1	1.2	
	4	6.2	0.3	0.1	1.0	
	5	4.3	0.1	0.1	3.2	
	6	10.6	0.7	0.1	5.5	
	7	3.2	0.0	0.0	0.3	
	8	4.5	0.1	0.0	2.1	

^a For chemotaxis each well contained 10,000 cells for WR19M.1 and its clones and 100,000 cells for all of the other cell lines and their clones. For phagocytosis IgG-coated red cells were incubated for 2 hr at a ratio of 40 red cells to 1 phagocytic cell. PU5-1R and WR19M.1 and its clones were tested on the same day; the other two cell lines and their clones were assayed on different days.

TABLE V
Selection of chemotactic cells^a

Selection	% of Cells Migrated			
	RAW264	WEHI-3 clone 1	PU5-1R	WR19M.1
1	27.5	2.3	0.8	1.4
2	40.1	7.9	1.8	1.3
3	51.8			
4	1.8			

^a Blindwell chemotaxis chambers and polycarbonate filters were sterilized by treatment with ethylene oxide. A 1:100 dilution of EAMS was added to the lower well of the chambers. For selections 2, 3, and 4 for RAW264, 500,000 cells ($1 \times 10^4/\text{mm}^2$ of filter) were added to the upper wells of chambers. In all other selections, 750,000 cells ($1.5 \times 10^4/\text{mm}^2$ of filter) were added to the upper wells. The cells were allowed to migrate for 4 hr. Cells on the top of the filter were removed by wiping with a cotton swab. Filters were then removed. Some filters were stained and counted to determine the number of migrated cells. Others were cultured in 25 cm² T-flasks in MEM-10%FCS with several medium changes. When the growing cells nearly covered the filters, they were scraped into the medium with a rubber policeman. When the cells nearly covered the surface of the flasks, they were harvested and tested and the selection was repeated.

DISCUSSION

The chemotactic properties of 5 macrophage cell lines were studied. One of the cell lines, WEHI-3, was different from the other 4 in attractant specificity, in the time course and in the cell density dependence of the response to EAMS. One possibility that would explain the different chemotactic properties of WEHI-3 is that this cell line is representative of an early stage of monocyte-macrophage differentiation. This possibility is strengthened by reports that WEHI-3 has other properties characteristic of monocytes (4, 26). In addition, 2 chemotactic properties, attractant specificity and the time course of the response to EAMS, have been reported for mouse resident peritoneal macrophages and mouse monocytes (21). When these data are compared with the chemotactic properties of the cell lines, 4 of the cell lines (RAW264, RAW309CR, PU5-1R, and WR19M.1) have chemotactic properties that are similar to macrophages and 1 of the cell lines (WEHI-3) has properties that are more similar to monocytes.

The time course of chemotaxis for RAW264, RAW309CR, PU5-1R, and WR19M.1 showed a lag of 1 to 2 hr before the cells began to migrate across the filter. However, after the initial lag migration was complete in 1 to 2 hr. The reasons for the lag are not known, but it may be related to the adaptation of the cells to the new environment and cell density on the polycarbonate filter, or it may be related to the metabolism of the attractant and the establishment of a sufficiently steep gradient. The lag is a function of the cells, not a physical limitation of the assay system, since other cells, for example human neutrophils (27), migrate within minutes. Mouse resident peritoneal macrophages also showed a similar lag of approximately 3 hr before migrating toward EAMS or LDCF (21).

In contrast, WEHI-3 did not have a detectable lag before migrating to EAMS. Migration was nearly complete in 2 hr. This is very similar to the time course of migration reported for mouse monocytes to another attractant, LDCF (21).

Differences in the capacity of the cell lines to recognize various attractants were found. Of the 5 cell lines only WEHI-3 did not migrate to C5a. This may be related to the finding that macrophages migrated to EAMS and monocytes did not (21). It has been reported that the major attractant in endotoxin-activated mouse serum is C5a (1). Therefore, 1 possibility is that monocytes and WEHI-3 do not have receptors for C5a. Alternatively, one or both of the cells may have C5a receptors, but the C5a specific pathway for transmission of the chemotactic signal is defective.

All of the cell lines, including WEHI-3, responded to EAMS. The fact that WEHI-3 responds to EAMS and not to C5a shows that EAMS contains attractants other than C5a. In addition, while WEHI-3 and monocytes share certain chemotactic properties, they differ in that only WEHI-3 responds to EAMS. This may indicate that WEHI-3 represents a differentiation stage between monocytes and macrophages.

Two of the cell lines, RAW309CR and PU5-1R, were tested for a chemotactic response to f-Met-Leu-Phe from 10^{-8} to 10^{-5} M and

neither responded. Mouse resident peritoneal macrophages also do not exhibit chemotaxis to f-Met-Leu-Phe (E. J. Leonard, unpublished results). f-Met-Leu-Phe is an attractant for leukocytes in other species (25). It would be of interest to know if the failure of mouse macrophages and macrophage cell lines to exhibit chemotaxis to f-Met-Leu-Phe is due to the absence of the receptor or to some other defect in the chemotactic response.

Unlike chemotaxis by human monocytes (28), chemotaxis by 4 of the cell lines showed a requirement for a certain density of cells. At low cell densities (below 10^3 cells/mm²) only a few cells migrated to EAMS. As the density of cells increased a larger fraction migrated, suggesting that the cells interact. A clone of WEHI-3 did not show a cell density dependence. Chemotaxis of mouse peritoneal macrophages at cell densities of 3×10^3 cells/mm² and above did not show a cell density dependence (2). However, the results of the experiments with the cell lines suggest that these densities may be too high to demonstrate a density dependence. Since macrophages accounted for only 30 to 40% of the cells, it is possible that other cell types could modify any cell density dependence that may exist.³ It is of interest to know if macrophages or monocytes show a density dependence since this could provide additional information on the macrophage and monocyte character of the cell lines. In addition, if chemotaxis by macrophages is dependent on cell density, this may be an important factor in the accumulation of macrophages at inflammatory sites.

For biochemical studies it is important to know that all of the cells can migrate. Three pieces of evidence suggest that the cell lines do not have a stable nonchemotactic subpopulation. First, in a few experiments essentially all of the cells have migrated. Second, all clones isolated from the cell lines were chemotactic. Third, it was not possible to select for a population of cells where 100% of the cells migrated. At sufficiently low cell densities, the failure of 100% of the cells to migrate is not a physical limitation of the assay system, since 100% of cultured bone marrow derived macrophages can migrate (W. Falk *et al.*, unpublished observations). These data suggest that each cell has a transient, nonchemotactic state and that specific conditions must be met before chemotaxis occurs.

The restrictive conditions for complete migration are possibly related to the variability of chemotaxis that was observed in our initial experiments. There was a small day to day variability and a much larger variability that occurred at unpredictable times. For example, most of the time 0 to 6% of the PU5-1R cells migrated to EAMS (Table I). However, on other occasions 37% (Fig. 1) and 39% of the cells migrated. Inversely, 23 to 27% of the RAW264 cells usually migrated; however, on a few occasions the response has been very weak (Table I and unpublished results). The large variability is a property of the cells since sister cultures also show the abnormal chemotaxis. This abnormality is transient and the normal response returns within a few passages. The reason for the abnormal chemotaxis is not known but some variables do not appear to be important. Variability was not associated with a change in serum, since the same lot of fetal calf serum was used throughout these experiments. The chemotactic response was not correlated with the viability of the cells, whether the cultures were mostly adherent or in suspension, or whether the cultures were in exponential growth or had recently become confluent. Under the standard chemotactic assay conditions the cells were incubated for a time (4 hr) sufficient for nearly all of the cells to migrate and at an EAMS concentration (1/100 dilution) that was not sensitive to small changes in concentration. Therefore, it is unlikely that these 2 variables contribute significantly to the variable chemotactic response. The large changes that occurred in the chemotactic response show that a cell line must be tested several times before it is concluded that it does not have a certain activity such as chemotaxis or phagocytosis.

The similarities of the cell lines to macrophages and monocytes make them good models to study macrophage and monocyte chemotaxis. The cells are easily grown and harvested. The addition of anticoagulants or other chemicals is not necessary. On the other

hand, drugs can be easily added to the cells and the effects can be determined in the absence of other cell types. The cells can be cloned and mutants can be isolated. These properties of the cell lines should make it easier to determine the biochemical mechanisms involved in chemotaxis.

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³ Recent studies have shown that mouse resident peritoneal macrophages exhibit a time and cell density dependent increase in responsiveness to chemoattractants (Leonard, E. J. and A. Skeel. *J. Reticuloendothel. Soc.* In press.