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# Development of Chemotaxis and Formyl Peptide Binding in Human Promyelocytic Leukemia Cell Line (HL60) (41369)

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*Abstract.* HL60 lack motile behavior in general, as shown by failure to undergo random migration, chemotaxis, or phagokinesis. Upon induction with 1% DMSO, HL60 undergo myeloid differentiation and display motile behavior as measured by these three assays. Random migration and chemotaxis first appear 48 hr after DMSO induction and increase in magnitude over a 6-day period. Although lower in absolute magnitude, the chemotactic response of induced HL60 is similar to mature PMN. DMSO-treated HL60 respond chemotactically to formylpeptides, pepstatin, bacterial supernatants, and ZAS but fail to respond to GHG or VGSG. HL60 and PMN also show similar dose–response curves to formyl peptides. Uninduced HL60 lack specific [$^3$H]FMLP binding. Specific [$^3$H]FMLP binding appears coincidental with chemotaxis after 48 hr in 1% DMSO and both increase in parallel throughout the induction time course. These data further support the role of specific membrane receptors and cell locomotion in general in the maturation of the chemotactic response.

HL60 is a human leukemia cell line established by Collins *et al.* (1) from peripheral blood leukocytes of a patient with acute promyelocytic leukemia. In response to various biological and chemical inducers, these immature cells develop many of the morphological, biochemical and functional properties characteristic of mature granulocytes (2–4) and macrophages (5). HL60 is an *in vitro* model suitable to study of the relationship between membrane receptors and development of the chemotactic response. Specific membrane receptors for various chemotaxins have been described on human neutrophils (6, 7). The binding affinity for a series of *N*-formyl oligopeptides correlates with the chemotactic potencies (6). Lysosomal enzyme release (8) and superoxide production (9) are also observed subsequent to receptor binding. Although the precise role of this receptor in mediating these cellular functions is unclear, receptor-mediated endocytosis may be important (10).

In this report, we show that uninduced HL60 fail to show random migration or chemotaxis. After induction with dimethyl sulfoxide (DMSO), HL60 cells undergo myeloid differentiation and develop chemotactic responsiveness to formylated peptides, pepstatin, zymosan activated serum (ZAS) and bacterial supernatants, but fail to respond to gly–his–gly or the eosinophilotactic peptide val–gly–ser–glu. Induced HL60 also develop specific binding of [$^3$H]F-met–leu–phe, while uninduced cells fail to demonstrate specific binding of this peptide. In addition, we have observed that the kinetics of appearance of specific [$^3$H]F-met–leu–phe binding parallels the appearance of chemotactic responsiveness to formylated peptides (these findings have been previously reported in abstract form (11)). Niedel *et al.* (12) also recently showed specific binding of another formylated peptide to HL60 cells. These observations further support the role of these membrane receptors in chemotaxis.

**Material and Methods.** *Cells.* The HL60 cells (initially obtained from K. A. Smith Dartmouth Medical School, Hanover, N.H.) were grown in RPMI supplemented with 10% heat-inactivated fetal calf serum, 4 m*M* L-glutamine, 200 U/ml penicillin, and 200 $\mu$g/ml streptomycin (GIBCO). Cells were seeded in plastic flasks at $2 \times 10^5$ cells/ml and cultured in the presence or absence of 1% (v/v) dimethyl



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sulfoxide (DMSO) for 1–6 days. Day 0 cells represent untreated cultures and Day 6 cells were incubated for 6 days in the presence of 1% DMSO. Cells were harvested at designated times and washed free of DMSO. Human polymorphonuclear leukocytes (PMN) were isolated from heparinized blood using Ficoll–Hypaque (Sigma, Winthrop) gradients and subjected to brief hypotonic lysis to remove erythrocytes. These preparations were 85–95% PMN as determined by Wright–Giemsa staining.

*Materials.* DMSO (Sigma, Grade I, lot 108C-0319) was used for all inductions. Stock solutions of *N*-formyl-met–phe (FMP), *N*-formyl-met–leu–phe (FMLP), and pepstatin (Sigma) in DMSO were diluted with saline such that the final concentration of DMSO never exceeded 0.1%. Gly–his–gly (GHG, Sigma) and val–gly–ser–glu (VGSG, Miles) were dissolved in saline. Supernatants from overnight cultures of *Escherichia coli* grown in tryptose phosphate broth were harvested and used undiluted. Zymosan-activated serum (ZAS) was prepared by incubating 25 mg of zymosan (Sigma) with 1 ml of fresh human serum for one hour at 37°.

*Chemotaxis assay.* Chemotaxis under agarose was performed using Eagle's minimal essential media with 10% fetal calf serum and 1.2% agarose (Litex) (13). Cells were resuspended in RPMI without serum and 6 $\mu$l containing $4-5 \times 10^5$ cells were added per well. Chemoattractants were added to adjacent wells and plates were incubated at 37° in 5% $CO_2$ for 3 hr. Plates were fixed and stained and then viewed with an overhead projector (Bausch and Lomb) at 50× magnification. Results are expressed as the distance (centimeters) to the leading front at 50× magnification migrated toward the chemotaxin (directed migration) and the distance moved in the opposite direction (random migration). The difference between these two numbers was used as a measurement of chemotaxis. All assays were performed in triplicate. All values are reported as the mean of three experiments ± standard deviation.

*[$^3$H]FMLP binding.* Formyl peptide binding was measured using *N*-formyl-methionyl – leucyl – [$^3$H]phenylalanine (New England Nuclear, specific activity 56.9 Ci/mmole) by the method of Nelson *et al.* (14). Briefly, $10^7$ cells in 1 ml of Dulbecco's phosphate-buffered saline (PBS) were mixed in plastic tubes with [$^3$H]FMLP at a final concentration of $2.9 \times 10^{-9}$ *M*. DMSO alone (0.1% final) or unlabeled FMLP ($2.9 \times 10^{-5}$ *M* final) was added to each tube as a measure of total peptide binding and nonspecific binding, respectively. Cells were incubated with mixing at 4° for one hour then sedimented by centrifugation in a Beckman microfuge for 30 sec. After removal of the supernatant, cell pellets were dissolved in Aquasol (New England Nuclear) and counted in a Packard liquid scintillation spectrophotometer. The difference in counts of samples with DMSO alone and those with excess unlabeled FMLP was defined as specific FMLP binding. The assay was performed in duplicate or triplicate determinations. All values are reported as the mean of three experiments ± standard deviation. All solutions and manipulations were at 4° to minimize receptor-mediated endocytosis and peptide hydrolysis.

**Results.** *Chemotaxis.* The ability of HL60 cells to exhibit motile behavior as a function of time of induction was studied. Uninduced HL60 failed to show random migration or directed migration toward FMP (Fig. 1) or a variety of the other chemotaxins tested over a wide range of concentrations. After induction with 1% DMSO, HL60 cells developed both random motility and directed migration toward FMP (Fig. 1). Motile behavior was first detectable in the agarose system on Day 2 of induction. Both random migration and chemotaxis toward FMP increased over the time of induction, subsequent to Day 2. The magnitude of random migration was similar for induced HL60 and PMN; 2.6 ± 0.3 cm for Day 6 HL60 and 2.8 ± 0.6 cm for PMN. However, directed migration toward $2 \times 10^{-5}$ *M* FMP was much lower in Day 6 HL60 (6.9 ± 0.7 cm) than in PMN (14.1 ± 0.7 cm). In addition to the observed increase in migration distance (leading front), the number of migrating cells per unit area

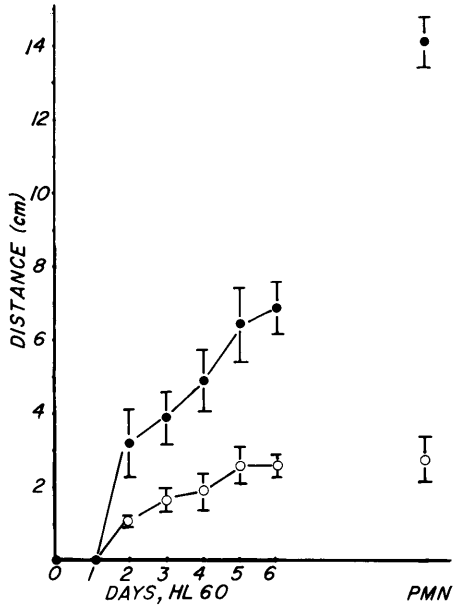


FIG. 1. Distance of migration of HL60 and PMN toward $2 \times 10^{-5}$ *M* FMP. HL60 were incubated with 1% DMSO for varied periods of time prior to harvesting. Directed migration (●), random migration (○). Mean of three experiments ± standard deviation.

increased over time of induction. This increases in motile behavior paralleled the increase in the number of mature myeloid cells in DMSO-treated cultures. Morphological examination of migrating cells demonstrated myelocytes and more mature forms.

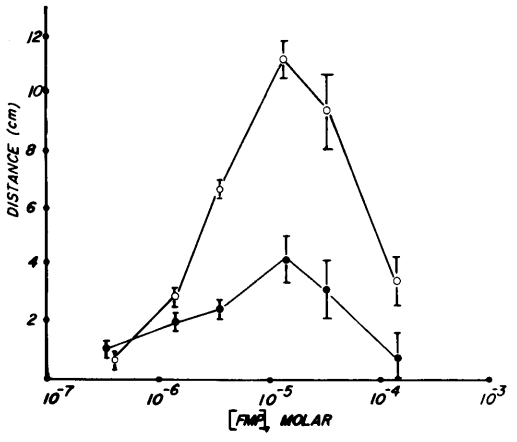


FIG. 2. Chemotaxis (directed minus random migration) as a function of FMP concentration. PMN (○), HL60 after 6 days exposure to 1% MSO (●). Mean of three experiments ± standard deviation.

To further characterize this chemotactic response, we studied the dose–response behavior of HL60 to chemotaxin concentration. Induced HL60 showd a similar dose–response relationship to varying concentrations of FMP as did PMN (Fig. 2). Both cell types exhibit a maximum response at $2 \times 10^{-5}$ *M* FMP, with inhibition of chemotaxis at higher concentrations. A similar curve was obtained using FMLP. The magnitude of the maximal response was similar for Day 6 HL60 using FMP (4.2 ± 0.8 cm) and FMLP (5.0 ± 0.8 cm) though the concentration required for maximum response was much lower for FMLP than for FMP ($1 \times 10^{-7}$ *M* vs $2 \times 10^{-5}$ *M*, respectively).

Similar to PMN, HL60 show chemotaxis to *N*-formyl peptides, pepstatin, *E. coli* supernatants, and ZAS (Table I). For all of these agents a chemotactic response was first observed on Day 2 and increased over the time of induction. The maximal response of HL60 cells was less than that of PMN for these chemoattractants. In the agarose system, induced HL60 and PMN failed to show chemotaxis toward GHG ($10^{-2}$ to $10^{-8}$ *M*) or toward the eosinophil-tactic peptide VGSG ($10^{-1}$ to $10^{-7}$ μg/ml) over a wide range of concentrations.

*[$^3$H]FMLP binding.* We assessed the ability of these cells to bind [$^3$H]FMLP. In this assay, PMN bound a total of 1912 ± 87 cpm of which 1005 ± 201 or 52% was due to specific binding of [$^3$H]FMLP. Day 0 HL60

TABLE I. CHEMOTAXIS OF HL60 AND PMN TO VARIOUS ATTRACTANTS[a]

| Attractants | Distance (cm) | |
|---|---|---|
| | HL60 Day 6 | PMN |
| FMP ($2 \times 10^{-5}$ *M*) | 4.2 ± 0.8 | 11.3 ± 0.6 |
| Pepstatin ($1 \times 10^{-5}$ *M*) | 6.5 ± 1.2 | 10.2 ± 0.6 |
| *E. coli* supernatant | 3.4 ± 0.4 | 5.5 ± 1.4 |
| Zymosan-activated serum | 1.8 ± 0.7 | 7.3 ± 1.3 |

[a] Chemotaxis (directed minus random migration) of HL60 after 6 days in the presence of 1% DMSO and PMN to various attractants. Values of FMP and pepstatin are given at the optimal concentration of peptide. Mean of three experiments ± standard deviation.

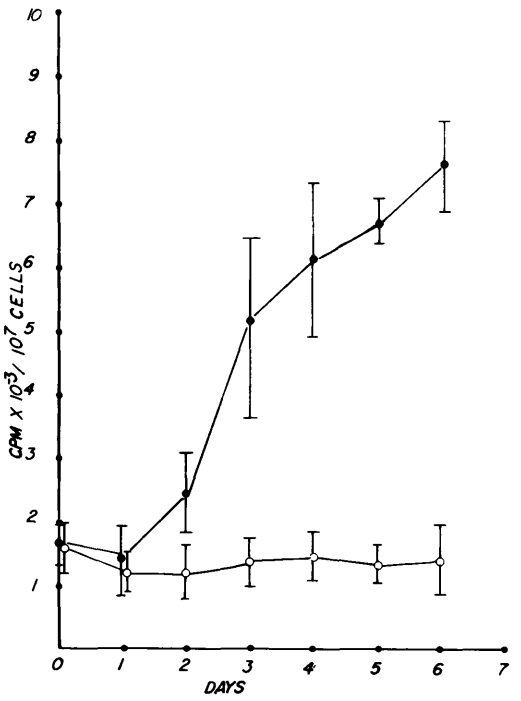


FIG. 3. [³H]FMLP binding to HL60 cells after varying times of exposure to 1% DMSO. Total cpm bound (●), nonspecific cpm bound (○). Mean of three experiments ± standard deviation.

cells bound a total of 1657 ± 273 cpm of [³H]FMLP of which 1597 ± 394 cpm was due to nonspecific binding; no significant specific binding was demonstrable. This lack of specific FMLP binding was consistent with the absence of chemotactic behavior in uninduced HL60. Upon induction with 1% DMSO, Day 6 HL60 bound a total of 7591 ± 660 cpm, of which 6179 ± 536 or 82% were due to specific [³H]FMLP binding. Over the induction time course, specific binding of [³H]FMLP was first demonstrable on Day 2 (Fig. 3). This specific chemotaxin binding on Day 2 coincides with the initial appearance of chemotaxis in these cells. Specific [³H]FMLP binding increased over time from 1214 ± 298 cpm or 50% of total counts bound on Day 2 to 6179 ± 536 cpm or 82% of total counts bound on Day 6. Nonspecific binding (counts not displaceable by 10,000-fold excess of unlabeled FMLP) remained essentially unchanged throughout the time course. The kinetics of appearance of specific [³H]FMLP binding paralleled the appearance of chemotaxis to formylated peptides during differentiation of HL60 cells *in vitro*.

On Day 6, HL60 cells bound 10.5 fmole/$10^6$ cells of [³H]FMLP with a calculated binding density of 6300 sites/cell assuming a homogeneous population of cells. However, the morphological cell types observed migrating under agarose represent only 20–40% of the total population. Assuming this subpopulation is responsible for the specific binding observed, the receptor density can then be estimated at 16,000–32,000 sites/cell. The value obtained for PMN is 1100 sites/cell in our assay system. These values are close to values reported for PMN (6) and HL60 induced with dimethylformamide (21) using [³H]FMLP.

**Discussion.** We have correlated chemotactic responsiveness and receptor binding in an *in vitro* model of myeloid differentiation (HL60). These studies provide further evidence for the myeloid properties of HL60 after DMSO induced differentiation.

Uninduced HL60 fail to demonstrate chemotactic behavior, consistent with observations reported by others (3, 10). The appearance and increase in the chemotactic response with induction can be attributed to the increase in the number of mature myeloid forms in the culture and hence the number of migrating cells. The increase in distance of migration (both random and directed) also suggests an increase in the overall rate of migration of these cells or in the size of a subpopulation of rapidly migrating cells as a function of differentiation.

Mature HL60 and PMN are responsive to a similar variety of attractants including *N*-formyl peptides (FMP, FMLP), *E. coli* supernatants, ZAS, and pepstatin. Pepstatin is an N-blocked hydrophobic pentapeptide proteinase inhibitor that has been reported to be chemotactic for PMN, monocytes, and eosinophils (15). Our results demonstrate the chemotactic property of this peptide in a myeloid cell line. Val–gly–ser–glu has been reported as a selective chemoattractant for eosinophils (16). The lack of significant chemotaxis of induced HL60 toward this tetrapeptide further supports the neutrophilic nature

of DMSO-induced HL60 differentiation. However, since the chemotactic response of HL60 to several classes of attractants is much lower than PMN in the agarose system, a small response to a weak attractant of PMN such as val–gly–ser–glu may be undetectable with HL60 cells. Filter chemotaxis, cell orientation, or labeled binding studies with these peptides would offer a more sensitive measure of this response.

The similar order of chemotactic potencies of formyl peptides (FMLP greater than FMP) in HL60 and PMN suggests similar specificity and hence structural relatedness of these chemotaxin binding sites on these two cell types. A characteristic dose–response curve with formyl peptides has been described in PMN with inhibition of chemotaxis occurring at high attractant concentrations. This inhibition may be related to functional loss of specific membrane receptors as a result of lysosomal enzyme release or receptor-mediated endocytosis (10, 17). Inhibition of chemotaxis by high concentrations of attractant was also seen in this myeloid cell line.

In the binding assay described using *N*-formyl-met–leu–[$^3$H]phe as a probe, we were unable to demonstrate significant receptor binding in uninduced HL60. In contrast, Niedel *et al.* (12) reported specific binding to uninduced HL60 using another formylated peptide, *N*-formyl-Nle-leu–phe-Nle-tyr $^{125}$I-lys; however, the magnitude of this binding varied widely. Using a fluorescent conjugate of this same peptide and video intensification microscopy these investigators showed that specific receptor binding was attributable to a small population of HL60 in uninduced cultures. The presence of this subpopulation in uninduced cultures was quite variable but paralleled the percentage of more mature cells in each culture. Several factors may contribute to failure to observe specific receptor binding of uninduced HL60 in our studies. Certain culture conditions, for example, serum source and lot, may favor the propagation of a more immature cell type in stock cultures. Second, differences in binding affinities of the receptor for a formyl hexapeptide and a different formyl tripeptide may contribute to differences in relative magnitude of receptor binding. Furthermore, the greater specific activity of the $^{125}$I-hexapeptide will allow detection of lower levels of specific binding than with the $^3$H-tripeptide.

Our findings of increased specific formyl peptide binding with DMSO induction of HL60 are in agreement with these same investigators (12). We observed a parallel appearance and increase in specific [$^3$H]-FMLP binding and chemotaxis to formyl peptides during differentiation of HL60. This finding further supports the role of this receptor in chemotaxis. In addition, the appearance of chemotaxis to ZAS also followed a similar time course. The chemotaxin in such preparations has been shown to be an activated fragment of the fifth component of complement (18). This attractant also has a specific membrane receptor on PMN, independent of the formyl peptide receptor (7). One may anticipate the parallel appearance of this receptor on HL60 as a function of differentiation.

The appearance of chemotactic behavior in this system is likely to be dependent upon more than appearance of these specific membrane receptors. A major cellular system necessary for cell locomotion as measured by random migration, phagokinesis, and chemotaxis is the cytoskeletal contractile apparatus. In the studies of phagokinesis by Albrecht-Buehler (19), certain cell types are observed to attach and spread on glass surfaces coated with colloidal gold particles. As the cells move on the substrata, they leave particle-free "phagokinetic" tracks, a process dependent on both phagocytosis and random locomotion (22). Phagokinesis was observed in induced HL60 but was not demonstrable in uninduced cells (Kowalchyk, Wood, and Douglas, unpublished observations). The lack of random migration, phagokinesis, and ability to adhere to glass surfaces we observed in uninduced HL60 and the appearance of these functions with differentiation is consistent with the concurrent maturation of such a cytoskeletal system in these cells. Hoffman-Liebermann and Sachs (20) have demonstrated an increase in actin content in a murine myeloid system as a func-

tion of differentiation. The appearance of chemotactic behavior in HL60 may therefore be dependent upon maturation of the cytoskeletal in addition to the appearance of specific receptors for chemotaxins. This study adds further support to the myeloid nature of DMSO-induced HL60 differentiation and the role of specific peptide receptors in chemotaxis.

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*Note added in proof.* During preparation of this manuscript, Fontana *et al.* (21) have reported similar results, which demonstrated specific FMLP binding and chemotaxis of HL60 after exposure to dimethyl formamide.

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