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Only the Chemotactic Subpopulation of Human Blood Monocytes Expresses Receptors for the Chemotactic Peptide *N*-Formylmethionyl-Leucyl-Phenylalanine

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Human peripheral blood monocytes comprise a subpopulation of 20 to 40% that is capable of responding to chemoattractants and a remaining subpopulation that cannot respond. We were able to obtain 99%-pure attractant-responsive monocytes by using a newly constructed separation chamber. The binding of the radioactive chemotactic peptide *N*-formylmethionyl-leucyl-[^{3}H]phenylalanine to migrating and nonmigrating populations was then studied. The binding was saturable at room temperature in the presence of azide. Saturation occurred at 5×10^{-8} M, and 50% of the maximal binding was obtained at 10^{-8} M, the concentration that induced optimal chemotaxis. The nonmigrating monocytes did not bind the peptide under the same conditions, which shows that at least one reason for a nonresponsiveness to chemotaxin is apparently a lack of receptors. By Scatchard analysis we calculated an equilibrium dissociation constant ranging from 23 to 37 nM; the number of binding sites per cell ranged from 64,000 to 77,000. The binding was very rapid. Fifty percent of the optimal binding occurred at 3.5 min, and equilibrium was reached after 20 to 30 min. Chemotactic deactivation of the monocytes reduced the number of available binding sites by 60%.

Chemotaxis of phagocytes may be important in the accumulation of effector cells at sites of inflammation. Receptor-ligand interactions have been the subject of several recent studies of the basic mechanisms of chemotaxis. Receptors for chemotactic peptides have been demonstrated for human (11, 15) and rabbit (1) polymorphonuclear leukocytes and for guinea pig macrophages (14). A receptor for the chemotactic fragment of the fifth component of complement, C5a, has been shown to be on the surface of human neutrophils (3). None of these studies addresses the possibility of a nonhomogeneity of the cell populations with respect to chemotactic responsiveness or receptor distribution. We have shown that only 20 to 40% of human blood monocytes migrate to chemoattractants and that each of these migrating cells can respond to several different attractants (6). This has recently been confirmed by the finding that only a subpopulation responds to chemotaxins by polarization (4). The human monocyte cell line U937 exhibits chemotaxis only when a surface receptor is expressed after induction by lymphokines (8, 12). The subject of this paper is the binding of *N*-formylmethionyl-leucyl-[^{3}H]phenylalanine (fMet-Leu-[^{3}H]Phe) to migrating and nonmigrating human monocyte populations. Using a newly developed method for isolating the migrating population, we show that only the migrating cells bind the chemotactic peptide. This is in contrast to the results of the accompanying paper (10), in which the binding of peptide to migrating human neutrophils is quantitatively indistinguishable from the binding of peptide to nonmigrating human neutrophils.

MATERIALS AND METHODS

Reagents. fMet-Leu-[^{3}H]Phe with a specific activity of 46.4 Ci/mmol was obtained from New England Nuclear Corp., Boston, Mass.; sodium azide and fMet-Leu-Phe were purchased from Sigma Chemical Co., St. Louis, Mo.

Cell preparation and separation by chemotaxis. Human blood mononuclear cells were isolated by the method of Boyum (2) and adjusted to 2×10^6 monocytes per ml. To separate migrating from nonmigrating monocytes, we used a newly constructed separation chamber (Neuro Probe Inc., Bethesda, Md.). As in our 48-well chamber (5), upper and lower compartments were separated by a Nuclepore membrane filter (25 by 80 mm), but the area was divided into only four large compartments (Fig. 1). The total filter area available for chemotaxis was 1,350 mm^2 (equal to the filter area of 170 wells of the 48-well chamber). The volumes of each of the four lower and upper compartments were 1.3 and 2.0 ml, respectively. The lower compartments were filled with an attractant solution; the upper compartments were filled with a cell suspen-

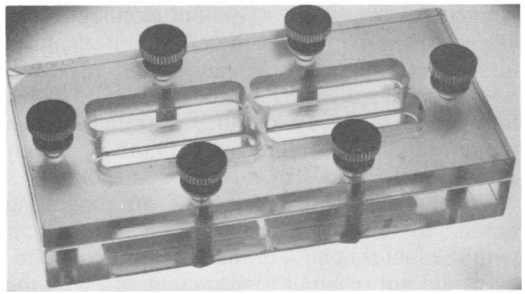

FIG. 1. Cell separation chamber. The chamber is composed of three parts. The lower plate contains four compartments for the attractant solution with a volume of 1.3 ml each. After the compartments are filled with the attractant solution, a Nuclepore filter sheet (25 by 80 mm) is applied. This is followed by a silicone gasket and the top plate, which is bolted in place. The cell suspension is added to the upper compartments, which have a volume of 2 ml each.

sion containing not more than 2×10^6 monocytes per ml. The chamber was then incubated for 70 min at 37°C in moist air containing 5% CO_2. By this time, attractant-responsive monocytes had migrated to the bottom surface of the filter, where they remained attached without falling into the lower compartment. The nonmigrating cells on the top of the filter were collected by resuspension with a pipette. The filter top was then wiped with a cotton swab to eliminate residual cells. The filter was removed and clamped, with the migrating-cell side exposed, to the inside of a 250-ml polypropylene tube with its neck cut off (no. 25350; Corning Glass Works, Corning, N.Y.). The migrating cells were rinsed off the filter with medium, washed once to remove traces of chemotactic factor and counted. The data on monocyte recovery and purity are presented below.

Monocyte deactivation. The monocytes were incubated for 45 min at 37°C with 5×10^{-7} M fMet-Leu-Phe at a cell concentration of 2×10^5 monocytes per ml of Gey balanced salt solution containing 2% bovine serum albumin. The cells were then washed twice and used for the binding assay.

Binding assay. The binding of tritiated fMet-Leu-Phe was assayed as described in the accompanying paper (10). The cells were suspended in Gey balanced salt solution containing 2% bovine serum albumin and 1 mM sodium azide to inhibit possible ligand internalization (14). Generally, 5×10^5 separated migrating or nonmigrating monocytes were used per assay point. The cells were incubated for 30 min at 24°C with concentrations of the radioactive peptide ranging from 10^{-9} to 10^{-7} M, with or without a 100-fold excess of the nonradioactive peptide. After incubation, the suspensions were filtered through Whatman glass fiber filters. The filters were washed with 10 ml of cold Hanks buffered salt solution containing 2% bovine serum albumin and were placed in scintillation vials with 10 ml of Ultrafluor scintillation fluid (National Diagnostics, Sommerville, N.J.). They were allowed to equilibrate for at least 3 h to obtain maximal counts. Nonspecific binding, defined as the amount of fMet-Leu-[^{3}H]Phe bound to the cells when a 100-fold excess of the nonradioactive peptide was present with the radioactive peptide, was usually less than 5% of the total radioactivity bound. Because of this low figure, all data are presented without correction for nonspecific binding.

RESULTS

Cell separation. No methods have previously been available to prepare pure monocyte suspensions suitable for chemotaxis assays. In these studies, we designed a separation chamber (Fig. 1) to obtain purified monocytes by chemotaxis, as described above. The approach was based on the fact that with Ficoll-Hypaque-separated cells (comprising monocytes, lymphocytes, and approximately 0.5% neutrophils), the cells that migrated through the pores of a Nuclepore filter toward an attractant were almost exclusively monocytes. The few neutrophils that migrated fell off the polyvinylpyrrolidone-coated filters (9). Thus, after 70 min of incubation, the attractant side of the filter was covered with monocytes, which were then rinsed off the filter relatively easily with little mechanical force. No residual cytoplasmic material was seen on the filter after it was stained. The purity of the migrating monocytes was 99 to 100%. No trypan blue-positive cells were seen, and no gross morphological changes were detected. Typically, 20 to 40% of the input monocytes were recovered from the filter bottom. This corresponds to the migration percentage for monocytes from normal human donors (6). The total yield of migrating and nonmigrating monocytes was 75 to 90% in a series of experiments. The attractant used for the separations was a 1:500 dilution of a C5a stock solution (6). After the separation procedure, the migrating monocytes responded to three different attractants, i.e., C5a, fMet-Leu-Phe, and leukocyte-derived chemotactic factor; the percentage of cells migrating was similar for each attractant. Only 2 to 8% of the nonmigrating monocytes that were recovered from the top of the chamber responded to attractant, and this residual migration was eliminated after a second separation step.

Binding of attractant to migrating and nonmigrating monocytes. The migrating and nonmigrating monocytes were incubated for 30 min at 24°C with concentrations of fMet-Leu-[^{3}H]Phe ranging from 10^{-9} to 10^{-7} M, with or without a 100-fold excess of the nonradioactive peptide. There were 5×10^5 monocytes per tube. A binding curve for the migrating monocytes is shown in Fig. 2. Saturation was reached at an attractant concentration of 5×10^{-8} M. Fifty-percent of the maximal binding occurred at 10^{-8} M, the concentration that induces the optimal monocyte chemotactic response. In eight ex-

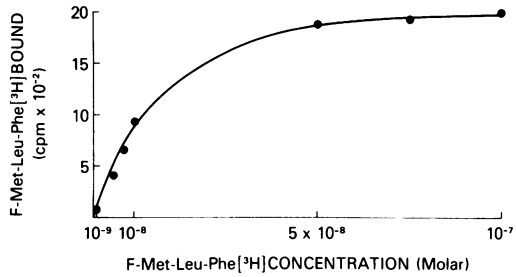

FIG. 2. Dose-response curve for the binding of fMet-Leu-[^{3}H]Phe to migrating monocytes separated by migration to C5a. The migrating monocytes, 5×10^5/assay point, were incubated with the indicated concentrations of the radioactive peptide in Gey balanced salt solution containing 2% bovine serum albumin, with and without a 100-fold excess of the unlabeled peptide. The total volume per tube was 1 ml. After incubation for 30 min at 24°C, the cells were centrifuged, and the supernatant was removed by suction. The cells were then resuspended and transferred onto glass fiber filters. The cells were washed with a large volume of medium, and the filter was then transferred to a scintillation vial and counted after at least 3 h of contact with the scintillation fluid. The values are for single determinations.

periments, 5×10^5 nonmigrating monocytes bound only 100 to 150 cpm, in contrast to 1,300 to 2,000 cpm for 5×10^5 migrating monocytes. Since at least 80% of the cells in the nonmigrating population were lymphocytes, the minimal binding shows that lymphocytes did not bind fMet-Leu-[^{3}H]Phe. The nonspecific binding, measured with a 100-fold excess of the unlabeled peptide, was less than 5% of the binding of the labeled peptide alone. The counts bound to the migrating monocytes represent 28 to 43 fmol of peptide per 10^5 monocytes. An analysis of the data by the method of Scatchard (13) yielded an equilibrium dissociation constant (K_D) ranging from 23 to 37 nM in different experiments; the number of binding sites per cell ranged from 64,000 to 77,000.

The binding data for migrating and unseparated monocytes were compared (Fig. 3). In this experiment, a Ficoll-Hypaque preparation of mononuclear cells was added to two separation chambers, one with and the other without C5a in the lower compartments. The cells that were added to the chamber without the attractant remained in the upper compartment, with the exception of a small number of cells that moved to the bottom of the filter by random migration; these upper-compartment cells are designated "unseparated." After a 70-min incubation period, we harvested the migrating monocytes from the bottom of the attractant chamber filter and the unseparated mononuclear cells from the upper compartments of the control chamber. Approximately 40% of the input monocytes migrated to the bottom of the attractant chamber filter. The binding of the radioactive attractant was determined over the 10^{-9} to 10^{-7} M range for aliquots of 5×10^5 monocytes from each cell preparation. The counts per minute bound at saturation for 5×10^5 unseparated monocytes were about half that for 5×10^5 migrating monocytes (Fig. 3). This difference is consistent with the fact that 60% of the unseparated monocytes did not respond to attractant and with the above finding that the nonmigrating monocytes bound little or no attractant.

Kinetics of binding. The migrating monocytes were incubated in 5×10^{-8} M radioactive peptide for the times indicated in Fig. 4, which is representative of three experiments. The binding reached equilibrium after about 20 min at room temperature; 50% of the maximal binding occurred at 3.5 min. The amount bound at 60 min was consistently slightly less than that bound at 20 to 30 min. In a different experiment, a 1,000-fold excess of the unlabeled peptide was added at various times after the addition of the labeled peptide, and 5 min thereafter the amount of bound radioactivity was determined. When the unlabeled peptide was added within 10 min after the addition of the labeled peptide, 50% of the bound counts were displaced. When the interval was 20 min or more, only 20% of the counts were displaced.

Effect of specific chemotactic deactivation on binding. The effect of specific chemotactic deactivation on binding was studied with unseparat-

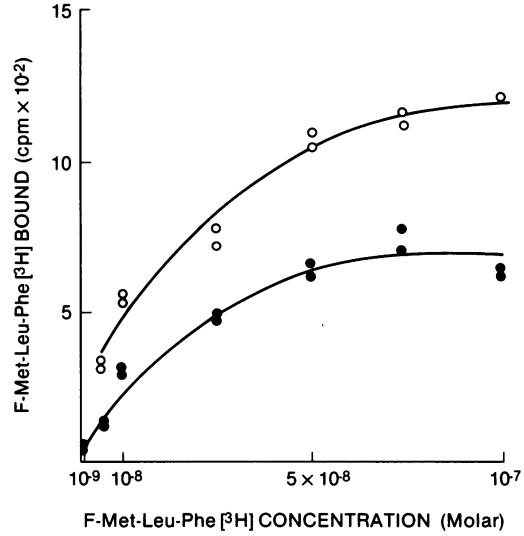

FIG. 3. Dose-response curves for the binding of fMet-Leu-[^{3}H]Phe to migrating (○) and unseparated (●) monocytes. The protocol was the same as for the experiment shown in Fig. 2.

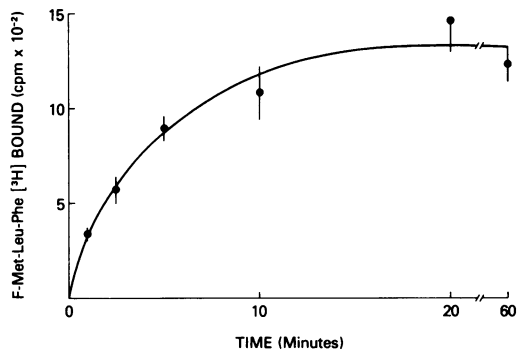

FIG. 4. Time course of binding. The experimental procedure was as described in the legend to Fig. 2. The concentration of fMet-Leu-[^{3}H]Phe was 5×10^{-8} M. The reaction was terminated at the indicated times by centrifugation and resuspension in cold medium. The points are means ± standard error of the mean for duplicate determinations.

ed cell mixtures. Since only the migrating cells bind the peptide, all changes measured can be attributed to the migrating population. Monocytes were deactivated with 5×10^{-7} M fMet-Leu-Phe (see above) under conditions that caused the deactivation of 65 to 90% of the cells (7). Control cells were incubated in medium without attractant. The cells were then washed, and the binding of fMet-Leu-[^{3}H]Phe was determined. The amount of radioactivity bound in 30 min by 2.5×10^6 control mononuclear cells was 3,100 cpm, whereas the same number of cells that had been incubated with deactivating concentrations of the peptide bound 990 cpm.

DISCUSSION

Using a method that separates migrating from nonmigrating populations of human blood monocytes, we have shown that the migrating cells bound chemotactic peptide with a high affinity (K_D of 23 to 37 nM). The nonmigrating population bound only small amounts of peptide, which could be attributed to the small residual number of migrating cells. Thus, the nonresponding cells appear to lack the chemotaxin receptor. Additional deficiencies in subsequent stages of the stimulus-response pathway have not been ruled out. A quantitative comparison of migrating and unseparated monocytes (Fig. 3) was consistent with the above findings and showed that the lack of peptide binding to the nonmigrated cells was not secondary to C5a exposure during the separation step.

It was shown recently that the human monocyte-like cell line U937 expresses peptide receptors only after stimulation with lymphokine-containing supernatants (12). Cells that did not respond to chemoattractant did not exhibit the receptor. The maximum number of binding sites for U937 was 33,000, and the K_D was 15 to 30 nM. The number of sites is about half that determined by us for human blood monocytes, which may be due to the fact that the proportion of migrating cells was not considered in the calculations for the cell line.

We have previously shown that the chemotactic deactivation of human monocytes is chemotaxin specific (6, 7). Deactivated cells are capable of responding normally to a different attractant, indicating that the migration machinery is intact and that the defect is at the level of the specific receptor, either in ligand binding or in postbinding transduction. In the present study, we found a decrease in the capacity to bind fMet-Leu-[^{3}H]Phe after chemotactic deactivation by fMet-Leu-Phe. This decrease in the capacity to bind new peptide shows that there is an alteration at the receptor, i.e., either continued occupancy by the deactivating peptide or receptor removal.

The detection of receptor appearance and disappearance may provide a tool for the investigation of monocyte maturation and the biochemical mechanisms of chemotaxis. For example, does the absence of peptide receptors on the nonmigrating human monocytes reflect a maturational stage of the cells? The question can now be approached experimentally. In this context, the finding in the accompanying paper (10) is of great interest. The binding of the chemotactic peptide to the nonmigrating neutrophils is indistinguishable from the binding to the unseparated population that contains migrating and nonmigrating cells. Thus, the deficiency in human neutrophils appears to be subsequent to receptor-ligand binding. An investigation of these two cell types offers the possibility of learning about the transduction of information after ligand binding. Finally, our findings show that great care must be taken in correlating biochemical data from whole populations with chemotactic responses, since only a fraction of the whole population may migrate.

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