Characterization of an Oligopeptide Chemoattractant Receptor on Human Blood Monocytes Using a New Radioligand

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The study of chemoattractant receptors on human monocytes had been limited by the lack of a radioligand suitable for use with the small numbers of cells routinely available from human donors. A new synthetic oligopeptide radioligand f$^{[35S]}$met-leu-phe, with a higher specific radioactivity than was available with the tritiated compound, was used to characterize a chemoattractant receptor on freshly isolated human blood monocytes. These cells bind f$^{[38S]}$met-leu-phe with a dissociation constant ($K_d$) of $30.2 \pm 5.6 \text{ nM}$ and contain $84,000 \pm 11,300$ receptors per cell. f$^{[35S]}$met-leu-phe does not bind specifically to blood lymphocytes. The specificity of the oligopeptide receptor on monocytes is indistinguishable from the oligopeptide chemoattractant receptor on human polymorphonuclear leukocytes. Using f$^{[35S]}$met-leu-phe, it will now be feasible to study the chemoattractant peptide receptor on small numbers of partially purified peripheral blood monocytes from patients with defects of immune function.

CHEMOTAXIS, the directed migration of cells along a concentration gradient of a chemoattractant, is a functional characteristic of human monocytes and is likely to be important in localizing these cells at sites of immunologic reactions. Indeed, abnormalities of monocyte chemotactic responsiveness have been associated with immunodeficiency and neoplastic diseases in humans. Receptors for chemoattractants have been found on human polymorphonuclear leukocytes (PMNs), monocytes, guinea pig macrophages, as well as on rabbit neutrophils. The ability to identify these receptors was dependent on the development of purified radioligands with high specific activities. A widely used radioligand for the study of chemoattractant receptors is N-formyl-methionyl-leucyl-phenylalanine (fMLP$^{[3H]}$), a potent chemoattractant with a specific radioactivity of approximately 47 Ci/m mole. This ligand is quite satisfactory for characterizing chemoattractant receptors on neutrophils, as these cells are easy to obtain in large numbers and in highly purified form. Human monocytes, however, are present in far lower numbers in blood than are granulocytes and are difficult to obtain in a high degree of purity free from contaminating lymphocytes; moreover, the specific activity of fMLP$^{[3H]}$ is not high enough to be practical for studying the chemoattractant receptor on small numbers of human monocytes. The iodinated hexapeptide f-nle-leu-phe-nle-f$^{[125I]}$tyr-lys (f-NLPN$^{[125I]}$-TL), which can be synthesized with a high specific radioactivity (ca. 450-1,700 Ci/m mole) has recently been used to study the oligopeptide chemoattractant receptor on adherent blood monocytes. However, $^{125I}$ has a short half-life, and the radio-ligand is not available commercially, making it impractical for routine laboratory use. Considering the potential importance of characterizing the chemotactic factor receptor on monocytes in humans with abnormal immune function, we have encouraged the synthesis of a suitable radioligand that would be commercially available. f$^{[35S]}$-met-leu-phe (f$^{[35S]}$MLP) has recently been synthesized by New England Nuclear Corporation (Boston, MA) with a specific activity of >200 Ci/m mole. After comparing the behavior of f$^{[35S]}$MLP with fMLP$^{[3H]}$ in previously standardized binding assays, we have studied the chemotactic peptide binding characteristics of human peripheral blood monocytes.

MATeRIALS AND METHODS

Chemotactic Peptides

fMLP$^{[3H]}$, with a specific activity of 46.7 Ci/m mole, and f$^{[35S]}$MLP, with a specific activity of 498 Ci/m mole (±10%), were obtained from New England Nuclear. Unlabeled fMP and fMLP were purchased from Sigma Chemical Co. (St. Louis, MO). f$^{[35S]}$MLP was identical to both unlabeled and tritiated FMLP by reverse phase high-pressure liquid chromatography using a hydrophobic C18 column and a methanol:acetic acid (0.1 N) solvent system. Unlabeled f-nle-leu-phe (fNLP), f-met-leu-tyr (fMLT) was obtained from Vega Biochemicals (Tucson, AZ).

Cell Preparation

Cells were isolated from heparinized (10 U/ml) peripheral blood of healthy human volunteers, diluted 1:1 with 3% (w/v) Dextran (TS00, Pharmacia Fine Chemicals, Piscataway, NJ) and the erythrocytes allowed to settle for 30 min at room temperature. The leukocyte-rich supernatant was then layered over a Ficoll-Hypaque (Bionetics Laboratory Products, Kensington, MD) density gradient and centrifuged at 400 g for 40 min at room temperature. PMN were isolated from the resulting pellet after 3 hypotonic lyses with 0.2% NaCl to eliminate RBC contamination; the pellet was resuspended.

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in incubation buffer (140 mM NaCl, 1.0 mM KH₂PO₄, 5 mM Na₂HPO₄, 0.5 mM MgCl₂, 0.15 mM CaCl₂, pH 7.4) at 25°C.

The mononuclear cell interface overlying the Ficoll-Hypaque was collected by gentle suction, washed twice, and finally suspended in incubation buffer. Cytocentrifuge slide preparations were stained with a modified Wright’s stain (Diff-Quik solutions, American Scientific Products) and for myeloperoxidase and the percentage of monocytes was determined (average 30% ± 10%). The cells were then diluted to a concentration of 2 x 10⁶ monocytes/ml for use in binding studies, unless otherwise stated.

Purified lymphocytes were prepared by suspending the interface cells in HBSS with 5% heat-inactivated fetal calf serum and passage over a Sephadex G10 (Pharmacia Fine Chemicals, Piscataway, NJ) column according to the method of Ly and Mishell.12 The resulting cells were then washed twice in incubation buffer. In all cases, cells and buffers were kept on ice and brought to 25°C just before use in the binding assay.

**Binding Assay**

One hundred microliters of buffer containing cells (2 x 10⁵ monocytes or 7 x 10⁶ PMN, unless otherwise stated) was added to 15 x 75 mm polypropylene tubes containing 25 μl of various concentrations of radiolabeled fMLP in incubation buffer and 25 μl of unlabeled formyl peptides or incubation buffer alone, then vortexed and incubated in a shaking water bath at 25°C for 40 min. Incubation was stopped by rapid filtration through Whatman GFC glass fiber filters and washed 4 times with 4.5 ml cold buffer. The filters were placed in 10 ml Lefkofluor (Research Products Int. Co., Mount Prospect, IL) and after at least 1 hr, radioactivity was determined. The solid line represents the computer-fitted line for the total ligand bound in this experiment.

**Computer Modeling**

Data from binding studies were analyzed by computer using SCTFIT, a program based on the principle of mass action ligand-receptor interactions.13 SCTFIT uses a nonlinear least squares, curve-fitting method, providing rigorous statistical analysis of binding data with determinations of the equilibrium dissociation constant (KD) and receptor number. The program also allows analysis of receptors that may exist in multiple affinity states.14

**RESULTS**

**Comparison of [35S]- and [3H]-Labeled fMLP Binding to Human PMNs**

Viable PMNs have been shown to possess specific binding sites for fMLP [3H]P on their plasma membranes.7 We compared [35S]MLP to fMLP [3H]P in binding experiments using intact PMNs. Computer analysis of both sets of data is best modeled to a single class of receptor sites. Using fMLP [3H]P, the average estimate of the KD, from 25 experiments, was previously shown to be 22.3 ± 2.4 nM with 55,760 ± 4,800 sites/cell.15 In two experiments using f[35S]MLP, the estimate for the KD was 20.7 ± 5.4 nM with 67,000 ± 14,600 sites/cell (Fig. 1). The determination of the KD and number of the receptors per cell was not significantly different with the two ligands. Koo et al. have recently described binding analysis of fMLP [3H]P to human PMN membrane preparations. In contrast to viable PMNs, binding of fMLP [3H]P to PMN membranes was best fitted to a model of two classes of receptors with different affinities.15 The detection of receptors in multiple affinity states in membrane preparations but not in viable cells is also seen in other hormone-receptor systems, including the dopaminergic and the alpha and beta adrenergic receptors.14

**Specificity of [35S]fMLP Binding to Monocytes and PMN**

Certain formylated oligopeptides other than fMLP also induce chemotaxis16,17 and compete with the binding of fMLP [3H]P to human PMN7 and guinea pig macrophages.9 The ability of these peptides to compete

![Fig. 1](image-url)
Fig. 2. $[^{35}\text{S}]\text{MLP}$ binding to human PMN membrane preparation. $[^{35}\text{S}]\text{MLP}$ was incubated with a standard amount of protein from a purified PMN membrane preparation, and the total (■) and nonspecific (○) binding was determined. The solid line represents the computer-modeled 2-site fit to the data, with $K_1$ (high) of $1.25 \pm 0.4 \text{nM}$ and $K_2$ (low) of $37.7 \pm 5.1 \text{nM}$. This is statistically better fitted ($p < 0.001$) to the data than the 1-site model, represented by the dotted line.

with fMLP binding can be taken as a measure of their affinity for the receptor and parallels their potencies as chemotactic agents. To be certain that f[35]SMLP bound to the chemotactic peptide receptor with the same specificity as fMLP, the ability of a series of formylated peptides to inhibit binding of f[35]SMLP to PMN and peripheral blood monocytes was tested. Each of the unlabeled formyl-peptides was added in different concentrations to a given volume of cells containing $5 \text{nM} f[^{35}\text{S}]\text{MLP}$, and the inhibition of specific binding, in the absence of any other competing ligand, was calculated. The potency of inhibition of f[35]SMLP binding to either PMNs (data not shown) or peripheral blood monocytes, as seen in Fig. 3, was

Monocyte Binding

Mononuclear cells isolated from whole blood by Ficoll-Hypaque were used in binding studies with both $[^{3}H]$- and $^{35}\text{S}$- labeled fMLP. Experiments were done with either 750,000 or 150,000 monocytes per assay and each incubation contained approximately $2.5 \times 10^6$ and $5 \times 10^6$ total cells, respectively. Figure 4 shows that with f[35]SMLP, either high or low numbers of monocytes could be used to obtain reproducible results with nonspecific binding, which remained less than 15% and usually less than 10% of the total binding.
Using fML[3H]P, highly variable data were obtained with the nonspecific component, which accounted for 60%–75% of total ligand bound to the lower number of cells (data not shown).

To show that f[35S]MLP was not binding specifically to the lymphocytes present, monocytes were selectively depleted from the mononuclear leukocyte preparation by passage over a Sephadex G10 column. The purified cells (>98% lymphocytes) and the original mononuclear leukocyte preparation (ca. 70% lymphocytes, 30% monocytes) were compared in binding experiments using the same total number of cells in each assay. As seen in Fig. 5, the total binding in the purified lymphocyte preparation was identical to the nonspecific binding component in the unpurified mononuclear leukocyte preparation. This confirms our previous observation that lymphocytes do not possess specific chemotactic peptide receptors,7 and therefore, only contribute to the nonspecific component of ligand binding in our assay.

With the preceding data established, detailed binding isotherms were performed using f[35S]MLP and the total number of monocytes standardized at 200,000/assay; the total number of cells per assay varied according to the percentage of monocytes in each preparation. A representative experiment from 3 experiments is shown in Fig. 6A. Computer modeling of these data was best fitted to a single class of receptors with a Kᵩ of 34.1 ± 4.5 nM and with 80,000 ± 10,400 receptors/cell. The average Kᵩ from 3 experiments was 30.2 ± 5.6 nM with 84,000 ± 11,300 receptors/cell. Using 8 data point binding isotherms, under these conditions, we found an average Kᵩ in 3 experiments of 28.5 ± 3.3 nM with 72,000 ± 7,800 receptors/cell.

**DISCUSSION**

Human monocytes adhered to plastic have been shown to express specific surface receptors for oligopeptide chemotactic factors,8 but detailed studies of these receptors in freshly isolated blood monocytes has been limited by the lack of a suitable ligand. The data presented here indicate that the newly synthesized f[35S]MLP is a satisfactory ligand for the study of chemoattractant receptors on human blood monocytes and should be particularly useful in analysis of this receptor in clinical situations. The behavior of the [3S]-ligand is analogous to fML[3H]P in binding to intact PMNs as well as to PMN membrane prepar-
tions. The specificity of the $^{35}$S compound, as well as its ability to identify high and low affinity binding sites in PMN membranes, was identical to fMLP.$^{[3H]}$. Because of the far higher specific radioactivity of the $^{35}$S compound, however, it was found to be useful for the study of chemoattractant binding to human monocyte preparations, whereas the tritiated compound was not. As few as 150,000 monocytes per assay provided excellent data using the $^{35}$S-labeled compound, with >85% specific binding always obtained. Standardizing the binding assay to contain 200,000 monocytes per replicate provided consistently reproducible binding data and allowed adequate studies of chemoattractant receptor binding using as little as 20 ml of whole blood. It was not necessary to purify the mononuclear leukocyte preparations beyond Ficoll-Hypaque or Lymphoprep gradients, as isolated lymphocytes neither bound f($^{35}$S)MLP nor interfered with the binding of this ligand to monocytes.

Based on three comprehensive binding experiments using mononuclear cells from three different individuals, it was estimated that human peripheral monocytes contain ca. 84,000 ± 11,300 receptors/cell with a $K_D$ of 30.2 ± 5.6 nM. Using more limited binding studies, such as would be possible in clinical studies where the amount of blood could be limiting, monocytes were found to contain 72,000 ± 7,800 receptors with a $K_D$ of 28.5 ± 3.3 nM. Neither of these values are significantly different from those obtained in the more detailed binding isotherms. These data compare with those previously obtained under the same conditions in binding of fMLP.$^{[3H]}$ to human PMNs that express 55,760 ± 4,800 receptors/cell, with a $K_D$ of 22.3 ± 2.4 nM.$^{15}$ Previous studies have indicated that only two-thirds of peripheral blood monocytes are capable of responding to chemoattractants by morphological polarization or chemotaxis$^{18,19}$ and that the nonresponsive monocytes do not bind chemoattractants.$^{19}$ This being the case, the number of receptors per chemotactically responsive monocyte would be approximately 120,000/cell.

In addition to being a useful ligand for the study of the monocyte chemoattractant receptor’s number and affinity in human disease states, the $^{35}$S-labeled compound may provide an excellent tool for the study of the dynamics of chemoattractant receptor processing following binding. Because of the high energy and low penetrance of the $\beta$ particles emitted by the $^{35}$S isotope, f($^{35}$S)MLP is a suitable ligand for use in light and electron microscopic autoradiographic studies of the receptor in ways not previously possible with tritiated or iodinated ligands.

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