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THE OLIGOPEPTIDE CHEMOTACTIC FACTOR RECEPTOR ON HUMAN POLYMORPHONUCLEAR LEUKOCYTE MEMBRANES EXISTS IN TWO AFFINITY STATES

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SUMMARY: The binding of the chemoattractant N-formyl-methionyl-leucyl-[^{3}H]phenylalanine to intact polymorphonuclear leukocytes and membrane preparations was analyzed by computer methods. Whole viable cells bind the chemoattractant with a single dissociation constant (K_D) of 22.3 ± 2.4 nM and contain an average of 55,000 receptors percell. In contrast, the binding data using membrane preparations were consistent with the presence of two classes of binding sites with average K_Ds of 0.53 ± 0.01 nM and 24.4 ± 1.2 nM. The high affinity receptors accounted for ca. 25% of the binding sites. Increasing the receptor occupancy did not affect the rate of dissociation of the ligand-receptor complex thus negative cooperativity is not a likely explanation for the complex binding isotherms. On the other hand, the dissociation kinetics did agree with the two affinity receptor model.

INTRODUCTION: Polymorphonuclear leukocytes (PMN)[1] are capable of migrating along minute concentration gradients of chemoattractants. Thus chemotaxis is likely to be a highly regulated biological response. Chemotaxis is initiated by the binding of chemotactic substance(s) to specific receptors located on the cell (1). Among a variety of chemicals that are chemotactically active are the synthetic formyloligopeptides. Using intact cells, receptors specific for the formyloligopeptides have been demonstrated on human PMNs and monocytes (2,3), rabbit peritoneal PMNs (4), guinea pig macrophages (5), and on two continuous human cell lines (6,7). Although the receptors are demonstrated to be on the cell surface,

[1]PMN, polymorphonuclear leukocytes.

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there have been no detailed studies of chemotactic factor receptors using preparations of membrane fractions.
A major advantage of analyzing membrane fractions is the ability to characterize receptors in the absence of interfering cellular processe such as endocytosis and exocytosis. Indeed, the availability of methods to study receptors in membranes has allowed major advances in the understanding of the regulation of adrenergic and cholinergic receptors (8). In the present study, we report the detailed binding characteristics of the oligopeptide chemotactic factor receptor on human PMN membrane preparations and compare these results with those obtained with intact PMNs.

MATERIALS AND METHODS: Chemotactic peptides: Formyl-methionyl-leucyl-[^{3}H]phenylalanine (FML[^{3}H]P)[2] with a specific activity of 46.7 Ci/mmol was purchased from New England Nuclear (Boston, Mass). Formyl-methionyl-leucyl-phenylalanine (FMLP)[3] was obtained from Sigma Chemical Co.(St. Louis, Mo).

Membrane Preparations: Human PMNs were isolated as previously described (2). The isolated PMNs were suspended in incubation buffer (140 mM NaCl, 1.0 mM KH_2PO_4, 5 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$) at a concentration of $8x10^6$/ml. When membrane preparations were required, the method of Davies et al. (9) was followed. The PMNs were resuspended in ice-cold buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.7) and were disrupted with a tissue homogenizer (20 sec. duration, repeated once). The suspension was centrifuged at 200 x g for 10 min at 4°C. The supernatant was decanted and centrifuged at 41,000 x g for 10 min at 4°C. The pellet was resuspended in incubation buffer at pH 7.4 using a teflon pestle. The final protein concentration was approximately 0.4 mg/ml in the membrane preparations.

FML[^{3}H]P Receptor-Binding Assay: 100 µl of buffer containing whole cells or membrane preparation was added to 15 x 75 mm polypropylene tubes containing 25 µl of FML[^{3}H]P (0.3-60 nM) and 25 µl of FMLP (6×10^{-4}M) or 25 µl of buffer. The membranes were incubated in a shaking water bath at 25°C. The incubation period was 30 minutes when membrane preparations were assayed and was extended to 60 min when whole cells were studied. Incubation was stopped by rapid filtration through Whatman GF/C filters followed by four washes each with 5 ml of ice-cold incubation buffer. Each determination was done in duplicate. The filters were counted in a Beckman liquid scintillation counter with a 60% coutning efficiency.

Formalin Fixation of Whole PMNs: Equal volume of 10% 0.01 M phosphate buffered formalin was mixed with an equal volume of a PMN

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[2] FML[^{3}H]P, N-formyl-methionyl-leucyl-[^{3}H]phenylalanine

[3] FMLP, N-formyl-methionyl-leucyl-pnenlylalanine

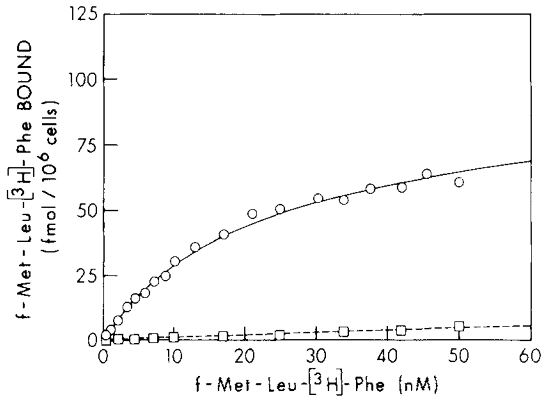

Figure 1. FML[^{3}H]P binding isotherms of intact PMNs. (o) - total ligand bound; (□) - nonspecific binding (defined as the amount of radioligand bound in the presence of 1000 fold excess of unlabeled ligand). The solid line represent the computer fitted line for the total binding and the dotted line represent nonspecific component. The K_D calculated from 25 experiments was 22.3 + 2.4 nM. The number of sites were calculated to be 55,760 ±4,800 sites per cell.

suspension at 8 x 10^6 cells/ml and then incubated at 0°C for 30 min. The cells were then washed twice with incubation buffer and the final concentration was adjusted to contain 8 x 10^6 cells/ml. Computer modeling: The experimental data was analyzed by computer using the method of Hancock, DeLean and Lefkowitz (10). This method of analysis is based on the principle of mass action ligand-receptor interactions and uses the non-linear least squares curve fitting method of Fletcher and Schrager (11). The model allows analysis of the binding of multiple ligands to multiple classes of receptors. In the present study, FML[^{3}H]P was the ligand, and the classes of binding sites were set equal to 1 or 2. The data was then fitted to 1 and 2 site models successively. The best fit was chosen on the basis of the lowest values of mean squares of residuals and a two-site model was accepted only when the fit of the data was significantly improved ($p<0.01$). The computer program allows the determination of the affinity constants and concentration of each class of receptors. The dissociation constants (K_D) are expressed as the geometric means ± SE (11).

RESULTS: Affinity and Concentration of FML[^{3}H]P Receptors on Intact Human PMNs. Intact PMNs have been shown to bind FML[^{3}H]P specifically (2). Figure 1 shows a saturation experiment using viable PMN cell preparations. The data was subjected to computer analysis and was found to be fitted to a single class of receptor sites with no significant improvement for a two site fit. The average dissociation constant (K_D) from 25 experiments was 22.3 ± 2.4 nM. The number of sites calculated from these experiments was 55,760 ± 4,800 (SEM) sites per cell.

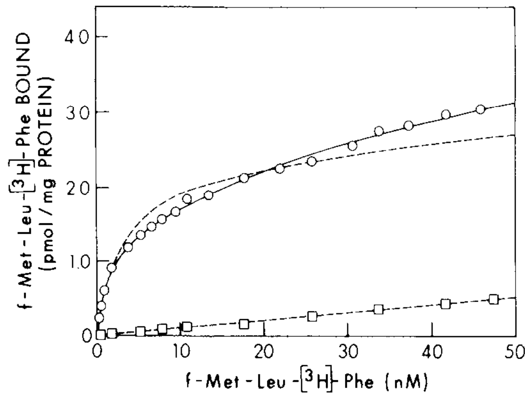

Figure 2. FML[^{3}H]P binding isotherms of PMN membrane preparations. The solid line shows a two site-fit to the data while the upper dotted line shows a one-site fit to the same data. The two-site fit was the significantly better fit with $p<0.001$. The lower dotted line represents nonspecific binding. The results for 20 such experiments yielded the following K_Ds: $K_H = 0.53 \pm 0.01$ nM, $K_L = 24.4 \pm 1.2$ nM. (o) total binding; (□) nonspecific binding

Affinity and Concentration of FML[^{3}H]P Receptors on PMN Membranes: PMN membrane preparations were found to bind FML[^{3}H]P specifically. Figure 2 shows that the nonspecific component was linear within the concentration range tested and that the specific binding approached saturation. Computer modeling of the binding data from each of 20 experiments indicated that it was significantly better fitted to a model with 2 classes of sites ($p\leq.001$). The dissociation constants for each of these 2 sites were 0.53 ± 0.01 nM and 24.4 ± 1.2 nM respectively. The concentration of the high affinity site varied in different membrane preparations from 10-30%. The dissociation constant of the lower affinity site detected in PMN membranes was not significantly different from the single K_D observed in viable, intact PMNs (22.3 nM for cells, 24.4 nM for membranes). The binding of the radioligand was reversible and the dissociation kinetics of the bound radioligand were consistent with the presence of binding site heterogeniety. Dissociation of the radioligand in the presence or absence of excess unlabeled ligand were comparable (Fig. 3).

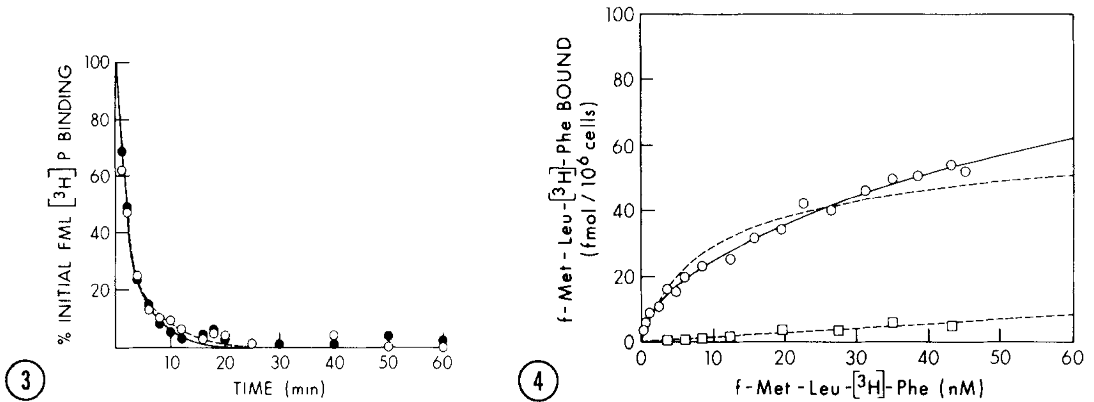

Figure 3. Time course of dissociation of bound FML[^{3}H]P from membrane preparations. Membrane preparations were incubated with 1 nM FML[^{3}H]P at 25°C for 30 min. The membranes were then sedimented at 4°C and the supernatant was removed and the membranes were resuspended in an equal volume of ice-cold buffer. The suspension was then diluted 100 fold with 25°C buffer with or without 10^{-5}M FMLP. Aliquots were filtered in duplicate at intervals and the radioactivity remaining bound to the membrane preparations was counted. Bound FML[^{3}H]P is expressed as a percentage of the amount of radioligand bound at time t = 0 min. All values are corrected for nonspecific binding. (●) dilution only; (o) dilution plus FMLP.

Figure 4. FML[^{3}H]P binding isotherms to formalin fixed PMNs. The solid line shows a two-site fit to the data while the upper dotted line represents the one-site fit to the same data. The two-site fit was the significantly better fit with $p < 0.001$. The lower dotted line represents nonspecific binding. The results from 8 experiments yielded the following K_Ds: $K_H = 0.55 \pm 0.3$ nM, $K_L = 18.6 \pm 3.1$ nM. (o) total binding; (□) nonspecific binding.

Affinity and Concentration of FML[^{3}H]P Receptors on Formalin Fixed Cells: Formalin fixed PMNs retained the ability to bind the radioligand FML[^{3}H]P. Computer analysis of the binding data revealed that in contrast with viable PMNs, formalin fixed PMNs bound FML[^{3}H]P with two different affinities (Fig. 4). The K_Ds of the two sites were 0.55 ± 0.3 nM and 18.6 ± 3.1 nM. The average number of receptors per cell for the high affinity site was 6.340 ± 950 and 39,000 ± 1.940 for the low affinity site. The two affinities were not significantly different from those measured in membrane preparations.

DISCUSSION: The data presented here show that specific receptors for chemoattractants are present on human PMNs as well as on PMN membrane preparations. The binding characteristics of the receptors on the membrane preparations differ however, from those found on whole PMNs. Viable PMNs bind FML[^{3}H]P with a single affinity of 22.3 $\pm$ 2.4 nM indicating one class of receptors averaging 55,000 sites per cell. In contrast to viable PMNs, binding of FML[^{3}H]P to PMN membrane preparations demonstrated heterogeneity. This heterogeneity of binding can be explained by two different models. One possible explanation of the experimental results is that the FML[^{3}H]P receptors display negative cooperativity. If this were the case then the rate of dissociationshould be a function of the fractional receptor occupancy with higher levels of receptor occupancy associated with more rapid dissociation (13). This however, was not the case since the rate of dissociation was not affected by the presence of a sufficient concentration of unlabeled ligand to occupy more than 99% of the receptors (Fig. 3).

An alternative interpretation of the data presented is that there are discrete classes of binding sites which differ in their affinities The observation of two binding affinities for the oligopeptide chemoattractant receptor in PMN membranes is not unprecedented in that heterogeneity of binding sites has been reported for peptide hormones such as insulin (14) and for many neurotransmitters (15-18).

The biological significance of the two affinity states of the chemotactic factor receptor is presently unknown, but the findings raise several interesting possibilities and testable hypotheses. One explanation is that the two binding affinities of the chemotactic factor receptor represent two distinct, non-interconvertible populations of binding sites. One could speculate that each of the receptor populations mediates different cellular responses to chemotactic factor stimulation. For example, chemotaxis is stimulated by far

lower concentrations of chemoattractants than is superoxide anion production or lysosomal enzyme secretion (19). Thus the possiblity exists that the high affinity receptor may mediate chemotaxis while the low affinity binding site mediates non-chemotactic functions. Another point which should be considered is that the two populations of sites could represent intracellular and extracellular receptors. Indeed, Fletcher and Gallin (20) have suggested that cryptic chemotactic factor receptors are present on human PMN specific granule membranes. Preparations used in our study did contain lysosomal granules as well as plasma membranes.

Rather than representing two distinct receptor classes it is also possible that the two binding affinities detected on human PMN membranes actually represent two affinity states of a single receptor population which under certain circumstances are interconvertible. Such a model has been postulated for the beta-adrenergic receptor (2]).

In any case, the data presented here provides the first direct evidence that chemotactic factor receptors on human PMNs exist in two affinity states. Whether the two affinities detected in the PMN membrane preparations represent two different receptors or different interconvertible states of the same receptor has not yet been determined. These studies will, however, provide the means for answering this question and for elucidating the metabolic processes which regulate the expression of the receptor and its role in initiating the biological responses stimulated by chemoattractants in leukocytes.

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