Demonstration of a Receptor on Rabbit Neutrophils For Chemotactic Peptides

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Received December 10,1976

SUMMARY: [^{3}H]formylNorleu-Leu-Phe, a potent leucocyte chemoattractant, binds specifically to a site on rabbit neutrophils with an affinity of 1.5×10^{-9} M. Other acylated peptide chemoattractants displace this binding at concentrations closely related to those levels required to elicit chemotaxis. The binding fulfills the major criteria for demonstration of specific receptor sites and indicates that a natural bacterial product and synthetic formyl-peptides produce chemotaxis by the same receptor mechanism.

INTRODUCTION

Leucocyte migration toward the site of bacterial invasion may in part be explained by the fact that bacteria release attractants for these cells (1). Leucocytes have been shown in vitro to respond chemotactically to a variety of structurally unrelated compounds, including denatured proteins (2), factors derived from complement (3), and bacterial products (4). Recently, we have shown that small synthetic N-formylmethionyl peptides which may be related to natural bacterial leucoattractants induce leucocyte chemotaxis (5). A systematic study of the chemotactic activities induced by a number of formylated peptides has led to the identification of several peptides which are active at extremely low (10^{-10} M) levels (6). Moreover, the strict structural requirements for chemotactic activity suggested that neutrophils have stereospecific receptors. Using techniques developed for the measurement of opiate and neurotransmitter receptors on brain membranes (7), we now report the demonstra-

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tion of membrane receptors on rabbit neutrophils which appear to mediate chemotaxis induced by both natural bacterial peptides and formylated synthetic peptides.

MATERIALS AND METHODS

All amino acid residues are of the L-configuration. [^{3}H]fNLLP* (13.5 Ci/mmole) was obtained by solid phase synthesis of N$^{\alpha}$-formylNorleu-Leu-Phe(Cl)OH and conversion by catalytic dehalogenation to N$^{\alpha}$-formylNorleu-Leu(p-tritio)Phe-OH according to Day et al. (unpublished results). Activated rabbit neutrophils were obtained 12 to 14 hours after the intraperitoneal injection of 0.1% glycogen as described previously (8). Other commercially available di- and tripeptides (Vega-Fox Research Plus and Schwarz-Mann) were formylated as previously described (5). Formylated and nonformylated oligopeptides including unlabeled fNLLP and NLLP** were obtained from R. Freer et al. (NIDR Contract DE-52477).

Chemotactic activities of peptides and natural attractants were assayed as previously described (9) by counting the cells adhering to the underside of a micropore filter separating two chambers, the upper one containing the cells and the lower the attractant. Cell counts were obtained with a hemocytometer in all experiments. In the standard binding assay, neutrophils (4.4×10^6 cells) which had been treated briefly with TPCK,*** obtained from Calbiochem, Gaithersburg, Md., (0.1 mM), were incubated in 2 ml of Gey's balanced salt solution (10) containing 50,000 cpm of [^{3}H]fNLLP (1.5 nM) at 0° for one hour. The incubation was begun by the addition of the cells and terminated by rapid filtration onto Whatman glass fiber filters (GF/B) attached to a low pressure chamber. Cells were washed with two 7 ml portions of ice-cold phosphate-buffered saline solution (pH 7.4, 0.02 M). The complete filtration procedure for each sample took less then 15 seconds. Filters were transferred to counting vials containing 10 ml of scintillation fluid, Aquasol, (New England Nuclear, Boston, Mass.) and liquid scintillation spectrophotometry was carried out with an efficiency of 40%.

Preparations of Other Cell Types

Platelets. Freshly drawn human blood containing sodium citrate-EDTA (12.6 mM disodium citrate, 7 mM disodium EDTA; Kafka et al., in preparation) was centrifuged for 5 minutes at 600 x g. Forty ml of the platelet-rich plasma (the low speed supernatant) were centrifuged at 10,000 x g for 10 minutes and the platelet-rich pellet was resuspended in 5 mls of Gey's medium. Aliquots (0.5 ml) which contained 1.5 mg of protein were assayed for binding in triplicate in the presence of fNLLP (10^{-6} M) or NLLP (10^{-6} M).

Red Cells. Heparinized rat blood (10 ml) was collected from decapitated rats and centrifuged for 5 minutes at 600 x g. The red cell-rich pellet was resuspended in 5 mls of Gey's medium and assayed as described for platelets.

Lymphocytes. A fraction rich in human lymphocytes (J. Tallman, C. Smith and C. Pert, in preparation) was obtained from the supernatant fluid which resulted after 50 mls of human blood were allowed to settle for 30 minutes. The supernatant fluid was centrifuged at 600 x g for 10 minutes and the pellet resuspended in 8 mls of Gey's medium without albumin. Aliquots (200 λ) which contained 0.5 mg of protein were assayed for specific chemoattractant receptor binding as described.

Brain Membranes. Brain membranes were prepared as described (11,12). Two ml aliquots containing about 3 mg of protein were assayed for specific binding as described.

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*[^{3}H]N$^{\alpha}$-formylNorleu-Leu-Phe-OH, **Norleu-Leu-Phe-OH, *** Tosyl-L-Phenylalanyl Chloromethane

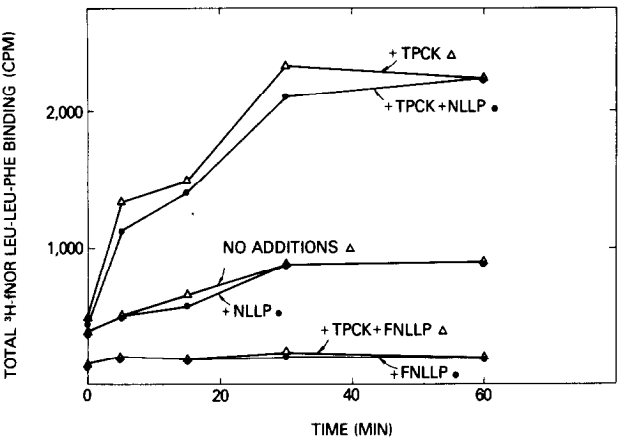

Figure 1. Specific binding of [^{3}H]formylNorleucyl-leucyl-phenylalanine to rabbit neutrophils as a function of time at 0°. Cells were exposed to ACK lysing buffer for 1 min at 0° to remove red cells as described in Methods. Each point is the mean of triplicate values with a SEM not greater than 10 percent. All samples contained labeled attractant.

RESULTS

In order to distinguish between binding of compounds due to specific receptor interactions and non-specific adsorption, we adopted the strategy of examining displacement of the labeled ligand by two compounds which are chemically similar, but biologically different. In this way, we were able to establish conditions maximizing the specific binding (Figure 1). The inclusion of the N-terminal formyl moiety has been shown to be critical for chemotactic activity since nonformylated compounds show chemotactic potencies lower by more than three orders of magnitude (6). Addition of the nonformylated peptide, NLLP (1×10^{-6} M), did not reduce the binding of [^{3}H]fNLLP. The same concentration of unlabeled fNLLP reduced the binding of radioactive compound by more than 90%. Non-specific, non-saturable binding which occurred in the presence of 1×10^{-6} M fNLLP was maximal at five minutes, the first time point tested, and did not alter with time. By contrast, binding of [^{3}H]fNLLP in the presence or absence of the non-formylated peptide developed gradually and reached a maximal level after thirty minutes.

Rabbit neutrophils contain a peptidase which is specific for aromatic amino acids (13). Incubations were therefore carried out at 0° to minimize proteolysis of peptide attractants. Even at this temperature, the addition of 10^{-4} M TPCK increased specific binding by more than 2-fold (Figure 1).

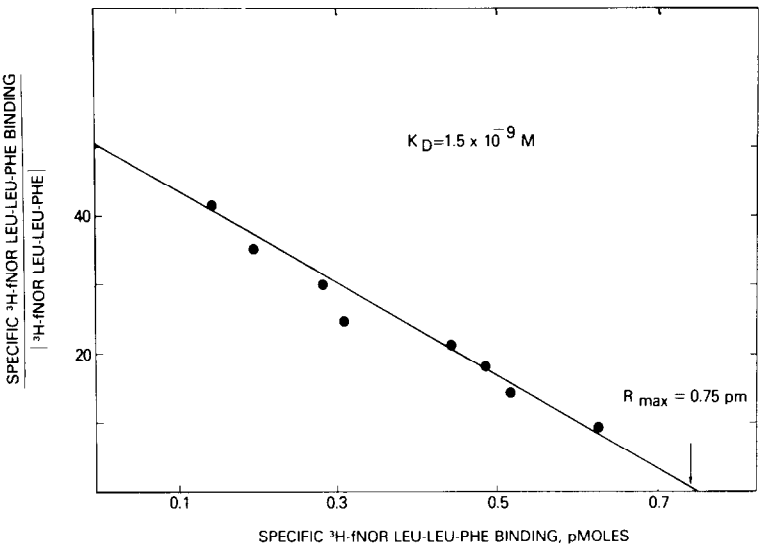

Figure 2. Scatchard plot (bound/free v. bound) of specific binding of [^{3}H]-formyl-Norleucyl-leucyl-phenylalanine to rabbit neutrophils at 0° as a function of the concentration of labeled ligand.

Therefore, incubations were routinely conducted for one hour at 0° with cells that had been treated with TPCK. Specific binding remained at maximal levels for two hours (not shown), indicating that equilibrium conditions were achieved. Specific binding was defined as the binding which occurred in the presence of NLLP (10^{-6} M) minus the binding which occurred in the presence of fNLLP (10^{-6} M).

Scatchard analysis of specific [^{3}H]fNLLP binding revealed the presence of a binding site with an affinity of 1.5×10^{-9} M (Figure 2). This value is close to that concentration of fNLLP required to elicit a half-maximal chemotactic response, 7×10^{-10} M (Day et al., unpublished results). At saturation, the 4.4×10^6 cells present in the incubation medium are capable of binding 0.75 pmoles of the [^{3}H]-ligand. Specific binding was linear with cell concentration over the range of 0 to 6.6×10^6 cells.

If we are indeed measuring a specific receptor responsible for mediating chemotaxis in neutrophils, it should be possible to demonstrate a close correlation between concentration of peptide required to displace 50% of the specific binding and the concentration of peptide required to elicit a half-maximal chemotactic response. This analysis is presented in Table 1. The three most potent attractants examined, formylMet-Leu-Phe, formylNorleu-Leu-Phe and formylMet-Met-Met-Met displace 50% of the specific binding

TABLE 1

Comparison of the Displacement of Specific [^{3}H]formylNorleu-Leu-Phe-Binding and Chemotactic Potencies of Synthetic Peptides

Peptide	Specific Receptor ID 50 (M)*	Chemotactic Activity ED 50 (M)**
fMet-Leu-Phe	3.3×10^{-10}	7.0×10^{-11}
fNorleu-Leu-Phe	3.4×10^{-9}	7×10^{-10}
fMet-Met-Met-Met	8.5×10^{-10}	3×10^{-7}
fMet-Leu	2.4×10^{-7}	4×10^{-7}
fMet-Leu-Arg	4.4×10^{-7}	3×10^{-6}
fMet-Leu-Glu	9×10^{-6}	1×10^{-6}
Norleu-Leu-Phe	4.2×10^{-5}	2×10^{-6}***
fMet-Asp	$>1 \times 10^{-3}$	non-attractant
fMet-Pro	$>1 \times 10^{-4}$***	non-attractant
fMet-His	$>1 \times 10^{-4}$***	weak attractant at 10^{-3} M

[^{3}H]formyl-Norleu-Leu-Phe was incubated with three concentrations of nonradioactive peptide as described in Methods. The concentration required to displace 50% of the specific binding (ID_{50}) was estimated from semi-log plots of percent inhibition of specific binding versus peptide concentration.

*Values are means of triplicate samples which varied less than 10 percent.

**From Showell, et al. (6), ED_{50} for chemotaxis is defined as the concentration of peptide giving 50% of the maximal activity.

***This is the lowest concentration at which significant chemotactic activity can be detected (5).

at the lowest concentrations listed (3×10^{-10} M to 5×10^{-9} M). Peptide attractants of medium potency (10^{-7} to 10^{-6} M range) displace 50% of specific binding at lower and similar concentrations. Chemotactically weak or inactive peptides fail to significantly inhibit specific [^{3}H]fNLLP binding even at 10^{-4} M, a concentration 10^5 times higher than the radiolabeled ligand.

Several experiments were performed which were designed to examine whether the observed specific "binding" actually represents transport and accumulation of radiolabelled ligand inside the cells. Cells which were homogenized and sonicated (Brinkmann Polytron, setting 5 for 1 minute), as well as cells which had been frozen and thawed repeatedly, showed similar levels of binding as intact cells. All binding activity appeared to be in the crude cell membrane fractions. The inhibitor, ouabain, failed to affect binding at a concentration of 10^{-5} M, which effectively inhibited chemotaxis without destroying viability.

TABLE 2

Comparison of Chemotactic Activity and Inhibition of Specific Receptor Binding of Natural Attractants

Attractant	Calculated Units of Chemotactic Activity (9)	Percent Inhibition of Specific Binding
Bacterial Factor*	270	88
	27	50
	4	0
Pronase-Treated Bacterial Factor (270 units)*	0	0
C5a**	31	0

*The bacterial factor was treated with pronase at 0.1 mg/ml at room temperature overnight. The incubations were terminated by immersion for 10 minutes in a boiling water bath. Undigested bacterial factor was treated identically. Values represent means of triplicate determinations which varied less than 15 percent.

**The chemotactic units, 31 for C5a and 27 for the bacterial factor are close to the maximal observed chemotactic response in the Boyden chamber assay (9).

In addition to rabbit neutrophils, several other cell preparations and tissue samples were examined for the presence of specific [^{3}H]fNLLP binding. Specific binding was not observed in rat red cells, human platelets, circulating human lymphocytes, or the synaptic membranes from rat brain.

Two natural chemotactic factors were examined for their ability to interact with the synthetic peptide chemotactic receptor (Table 2). C5a, a natural attractant derived during the serum complement cascade process failed to alter specific binding even in concentrations two times higher than that required to elicit maximal chemotaxis. A partially purified low molecular weight attractant obtained from E. coli culture media probably peptide in nature (9), inhibited specific binding by 50 percent at concentrations required to give a good chemotactic response. Also, treatment of this bacterial factor with pronase resulted in a concurrent loss of ability to induce chemotaxis and ability to displace specific binding. Presumably, this factor is a peptide which interacts at the same site as the synthetic attractants.

DISCUSSION

We have demonstrated that [^{3}H]formyl-Norleu-Leu-Phe binding to rabbit neutrophils is a saturable, time-dependent process. At 0°C, at least 90%

of the binding appears to be specific, i.e., displaceable by a potent chemotactic peptide but unaffected by the same concentration of its weakly chemotactic nonformylated peptide analog. Close correlation exists between the abilities of nine peptides examined to displace specific binding and their abilities to produce leucocyte chemotaxis, despite the fact that different conditions were used in the binding and chemotactic assays. These findings suggest that the absolute concentrations of peptide required to occupy 50% of the receptors is similar to that required to elicit a half-maximal chemotactic response. Specific binding sites did not appear to be present in several other tissues, suggesting that the distribution of receptors is limited to chemotactically active cell types and providing further evidence of binding specificity. It is possible to calculate the mean number of receptors per cell from maximal binding assuming an equimolecular ligand-receptor complex. The calculation of 10^5 receptors per cell is a figure of the same order of magnitude as that obtained for the number of β-adrenergic receptors on human lymphocytes (unpublished results) and opiate receptors on one neuroblastoma cell line (14).

The chemotactic peptide binding activity does not appear to be the result of active transport but rather is likely to be the property of a membrane component, since cell disruption by freezing and thawing or homogenization had little effect on binding.

It appears that a specific chemotactic receptor exists on rabbit neutrophil membranes which mediates chemotaxis induced by both natural and synthetic peptides. In addition, from unpublished results it appears that this receptor has optical specificity for peptide attractants since at equal concentrations formyl-D-Phe-DMet fails to affect chemotaxis or interact with peptide chemotactic receptors, while its enantiomer, formyl-L-Phe-L-Met has significant effects on chemotaxis and specific receptor binding. We have no evidence that C5a-induced chemotaxis operates by this receptor since C5a failed to interact with the chemotactic peptide receptor at concentrations above those shown to elicit chemotaxis. This "chemotactic peptide receptor" may also mediate other cellular events. In particular, these low molecular weight peptides induce lysosomal enzyme release from neutrophils (6) and histamine release from basophils (15) at concentrations which correlate closely with their chemotactic potencies. The method described here for measuring peptide attractant receptors can be used for detailed study of structure-activity relationships.

ACKNOWLEDGEMENT

The authors are grateful to Mrs. Dawn McBrien for her gracious and efficient preparation of the manuscript.

REFERENCES

1. Ward, P.A., Cochrane, C.G., and Müller-Eberhard, H.J. (1966) Immunology, 11: 141-146.
2. Wilkinson, P.C. (1974) In Chemotaxis and Inflammation. Churchill Livingston, Edinburgh, London, 54.
3. Snyderman, R., Phillips, J., and Mergenhagen, S.E. (1970) Infec. Immunity, 1: 521-525.
4. Keller, H.V. and Sorkin, E. (1967) Int. Arch. Allergy, 31: 505-517.
5. Schiffmann, E., Corcoran, B.A., and Wahl, S.M. (1975) Proc. Natl. Acad. Sci., 72: 1059-1062.
6. Showell, H.J., Freer, R.J., Zigmond, S.H., Schiffmann, E., Aswanikumar, S., Corcoran, B.A., and Becker, E.L. (1976) J. Exp. Med., 143: 1154-1169.
7. Pert, C. and Snyder, S.H. (1973) Science, 179: 1011-1015.
8. Tempel, T.R., Snyderman, R., Jordan, H.V., and Mergendagen, S.E. (1970) J. Peridontol., 41: 3/71-12/80.
9. Schiffmann, E., Showell, H.J., Corcoran, B.A., Ward, P.A., Smith, E., and Becker, E.L. (1975), J. Immunol., 114: 1831-1837.
10. Gey, G.O., and Gey, M.K. (1936) Am. J. Cancer, 27: 45-76.
11. Pert, C.B., Pert, A., Chang, J.-K., Fong, B.T.W. (1976) Science, 194: 330-332.
12. Pert, C.B. and Snyder, S.H. (1974) Mol. Pharmacol., 10: 868-879.
13. Aswanikumar, S., Schiffmann, E., Corcoran, B.A., and Wahl, S.M. (1976) Proc. Natl. Acad. Sci., 73: 2439-2442.
14. Klee, W. and Nirenberg, M. (1974), Proc. Natl. Acad. Sci., 71: 3474-3477.
15. Hook, W.A., Schiffmann, E., Aswanikumar, S., and Siraganian, R.P. (1976), J. Immunol., 117: 594-596.