SOME CHARACTERISTICS OF THE NEUTROPHIL RECEPTOR FOR CHEMOTACTIC PEPTIDES

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1. Introduction

Formylated peptides have been shown to be potent chemoattractants for leukocytes [1,2]. With the aid of an intrinsically labelled chemoattractant, f-Nle–Leu–[^{3}H]Phe (fNL[^{3}H]P) [3], a high affinity binding site (K_d 1.5 nM) was demonstrated for formylated peptides on rabbit neutrophils [4]. The no. binding sites/cell was estimated to be ~10^5. In [5], 0.7 × 10^5 sites/cell were estimated. This putative receptor, in addition to binding a number of peptide agonists and antagonists, interacted with a partially purified chemotactic factor produced by *Escherichia coli* [4]. Others have demonstrated in human cells a similar receptor for chemotactic peptides [6,7].

Here we present some characteristics of the chemoattractant binding site on the rabbit neutrophil. In an approach similar to that used to characterize other receptors such as the opiate [8] and β-adrenergic [9] binding sites, we have investigated the correlation between binding and biological activity for typical agonists and antagonists, the stereospecificity of the receptor, the effects of pH and ionic environment upon binding, and the chemical nature of the binding sites.

2. Materials and methods

Synthetic peptide attractants, both labelled and unlabelled, were obtained under NIDR contract DE-52477 and were composed of L-amino acids unless otherwise stated. f-L-Met–D-Leu–L-Phe and the enantiomers of Boc-Phe–Leu–Phe–Leu–Phe were synthesized by J. B. and E. G.. The following chemicals were obtained from commercial sources: lectins; enzymes; gangliosides; dithiothreitol; tosyl-L-phenylalanyl chloromethane (TPCK); tosyl-L-lysyl chloromethane (TLCK); Cupferron, *N*-ethyl maleimide; *N*-benzoyl tyrosine ethyl ester (BTEE); *p*-mercuribenzoate (pMB); and α,α-dipyridyl.

Rabbit peritoneal exudate cells were obtained as in [10]. Particulate preparations for binding assays were prepared by disrupting frozen and thawed cells with the use of a Brinkman Polytron as in [4]. Chemotaxis was assayed in a modified Boyden

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chamber containing a 5 μm Millipore filter [10]. Release of lysosomal enzymes in response to the presence of an attractant was determined as in [2]. The binding assay was performed as previously described, using f-Nle–Leu–[^{3}H]Phe (fNLL[^{3}H]P) and whole cells or particulate fractions [4]. Gey's balanced salt solution (pH 7.4) 2 ml, containing a suspension of 4.4×10^6 cells or their particulate equivalent, 0.2 mol TPCK, 2.0 pmol (50 000 cpm) of fNLL[^{3}H]P and, in some cases, a given concentration of the competing peptide were incubated at 4°C for 1 h. The cells were then rapidly filtered onto Whatman glass fiber filters (G F/B) under vacuum and washed rapidly with two 7 ml portions of cold 0.02 M phosphate-buffered saline, pH 7.4 (PBS). After drying, the filters were counted by scintillation spectrometry.

Cells were incubated for 30 min at 37°C with a given concentration of enzyme in Gey's buffer to determine the effect of enzymatic pretreatment of cells upon their subsequent ability to bind fNLL[^{3}H]P. The cells were then washed free of enzyme, resuspended in Gey's buffer and assayed for binding of labelled attractant [4]. Cell viability after enzymatic treatment was assessed by determining trypan blue dye exclusion.

3. Results

3.1. *Effect of proteolytic inhibitors upon binding of fNLL[^{3}H]P to whole cells*

We have demonstrated a protease activity in leukotaxis, with specificity for aromatic amino acid residues [11]. An enhanced binding of fNLL[^{3}H]P to neutrophil receptors was found to occur in the presence of the chymotryptic inhibitor TPCK [4]. We have examined the effect of chymotryptic and tryptic inhibitors and synthetic substrates for trypsin and chymotrypsin upon the specific binding* of fNLL[^{3}H]P to determine whether proteolysis alters the interaction of ligand with receptor. Table 1 shows that 0.1 mM TPCK, but not TLCK stimulated the binding of a labelled substrate by 2-fold. Also TPCK, in contrast to TLCK, markedly inhibited chemotaxis. In [11] TPCK was more effective than TLCK in inhibiting hydrolysis of a labelled attractant. BTEE, a chymotryptic substrate, did not appreciably affect binding but did depress chemotaxis. The tryptic substrate, tosyl-arginylchloromethane (TAME, not shown), at 0.1 mM did not affect binding or chemotaxis. We have also found (not shown) that the presence of TPCK prevented a small degree of hydrolysis (≤10%) of labelled attractant by cells alone. TLCK did not inhibit this hydrolysis, which occurred at 4°C during the binding procedure. These results suggest that a chymotryptic enzyme, perhaps closely associated with the binding site, may be required for chemotaxis and that its irreversible inhibition may contribute to enhanced binding of the attractant by preventing the latter's hydrolysis.

Table 1
Effects of protease inhibitors on chemotaxis and specific binding of fNLL[^{3}H]P

Inhibitor	Percent inhibition[a,b] of chemotaxis	Percent specific binding[b]
None	0	100
TPCK (0.1 mM)	100	191
TLCK (0.1 mM)	12	109
BTEE (0.1 mM)	99	93

[a] Standard conditions as in section 2 were employed for both chemotaxis and binding

[b] The values are means of triplicate measurements that varied <10%; 3 experiments were done

3.2. *Correlation between binding of synthetic peptides and their effects upon neutrophil functions*

We had provided evidence for the physiological significance of this receptor in that we showed a correlation between the ID_{50} of a peptide, that concentration required to decrease by 50% the specific binding of the labelled attractant to the receptor, and its chemotactic ED_{50}, that concentration which gives 50% of the maximal chemotactic response [4]. Extending these observations (table 2) we show a correlation not only between the ID_{50} and chemotactic ED_{50} values for a number of typical peptides but also between inhibition of binding of fNLL[^{3}H]P and release of lysosomal enzymes. The values for enzyme release and inhibition of binding appear to be closer than those for chemotaxis and inhibition of binding. For example, for f-Met–Leu–Phe the ID_{50} is 3.3×10^{-10} M, ED_{50} for the enzyme release is

* Specific binding is the difference between the total binding and the non-specific binding which occurred in the presence of excess (1 μM) unlabelled fNLLP

Table 2
Comparison of the displacement of specific binding of fNLL[^{3}H]P, chemotactic potencies and lysosomal enzyme releasing capacities of typical synthetic peptides

Peptide	Specific receptor ID_{50} (M)[a]	Chemotactic activity ED_{50} (M)[b]	Lysozyme releasing activity ED_{50} (M)
f-Met–Leu–Phe	3.3×10^{-10}	7.0×10^{-11}	2.4×10^{-10}
f-Nle–Leu–Phe[c]	3.4×10^{-9}	7.0×10^{-10}	1.5×10^{-9}
f-Met–Phe–Met	6.4×10^{-8}	1.5×10^{-9}	2.6×10^{-8}
f-CycloLeu–Leu–Phe[c]	9.2×10^{-6}	4.6×10^{-7}	2.8×10^{-6}
f-Gly–Leu–Phe	1.3×10^{-5}	4.3×10^{-6}	7.6×10^{-6}

[a] ID_{50} is the concentration of peptide required to displace 50% of the specific binding of the labelled attractant
[b] ED_{50} is the concentration of peptide required to give 50% of the maximal chemotactic activity
[c] ED_{50} is the concentration of peptide required to give 50% of the maximal release of lysosomal enzyme

fNLL[^{3}H]P was incubated with 5 concentrations of non-radioactive peptides (section 2) The concentration required to displace 50% of specific binding was estimated from plots of % inhibition of specific binding peptide concentration. Values are means of triplicate values varying <10%

2.4×10^{-10} M, and the chemotactic ED_{50} is 7.0×10^{-11} M. The results indicate that binding of a peptide is correlated closely with its ability to induce two biological responses in the neutrophil and that more binding is required to produce half-maximal enzyme release than is necessary for half-maximal chemotaxis. This correlation is observed for peptides that display a range of activities extending over 5 orders of magnitude.

3.3. *Stereospecificity of the receptor*

Since other well characterized receptors exhibited stereospecificity for their respective ligands [8,9] we determined whether this receptor responded stereospecifically. Using a number of stereoisomers (table 3) it was found that f-L-Met–L-Leu–L-Phe was both a better inhibitor of specific binding (ID_{50} = 3.8×10^{-10} M) and a more active chemotactic agonist (ED_{50} = 7.6×10^{-11} M) than f-L-Met–D-Leu–L-Phe

Table 3
Effects of diastereoisomeric peptides upon neutrophil functions and binding of fNLL[^{3}H]P to neutrophil receptor

Peptide	Specific binding[a,b] ID_{50} (M)	Chemotactic activity ED_{50} (M)	Lysozyme releasing activity ED_{50} (M)
f-L-Met–L-Leu–L-Phe	3.8×10^{-10}	7.6×10^{-11}	3.2×10^{-10}
f-L-Met–D-Leu–L-Phe	8×10^{-8}	6.0×10^{-8}	4.0×10^{-8}
Boc-L-Phe–L-Leu–L-Phe–L-Leu–L-Phe	6.8×10^{-8}	1.6×10^{-5}[c]	2.6×10^{-7}[c]
Boc-D-Phe–D-Leu–D-Phe–D-Leu–D-Phe	3.7×10^{-7}	1.8×10^{-5}[c]	3.6×10^{-7}[c]

[a] These values were obtained from dose-inhibition plots of varying concentrations of peptides in the presence of the labelled ligand
[b] Values are means of triplicate measurements that varied <10%; 3 experiments were done
[c] These values are for the inhibition exerted by the antagonists (Boc compounds) upon chemotaxis and enzyme release in response to a maximally stimulating concentration of agonist (f-L-Met–L-Leu–L-Phe)

Standard conditions as described in section 2, were employed for both chemotaxis, enzyme release and binding

(ID_{50} = 8 X 10^{-8} M; ED_{50} = 6.0 X 10^{-8} M). Boc-L-Phe–L-Leu–L-Phe–L-Leu–L-Phe was a more effective competitor (ID_{50} = 6.8 X 10^{-8} M) than its enantiomer (ID_{50} = 3.7 X 10^{-7} M). However, they were equally effective chemotactic antagonists of relatively low activity (10^{-5} M). The poor antagonism of these inhibitors on chemotaxis compared to their effect upon binding could be the result of their degradation during the chemotactic assay period (2 h at 37°C). We have found (unpublished) that after such treatment in buffer alone both compounds lost potency in inhibiting binding to the receptor. These results are in contrast to the more effective inhibition by the antagonists upon release of lysosomal enzymes. These were observed after an incubation period of a only few minutes.

3.4. *Effect of pH and cations upon specific binding in cell particulate preparations*

Using a particulate cell preparation to control microenvironmental pH and levels of ions, we studied the effect of pH and different cations upon binding. Over a pH range from 6–9 optimal binding occurred from pH 6.5–7.0 (fig.1). An optimal chemotactic response was also observed within the same range (not shown). However, we could not observe appreciable changes in binding at pH 7.0 as a function of certain monovalent or divalent cation levels. Indeed the presence (not shown) of either Na^+ or K^+ up to 0.2 M or of each of 3 divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) up to 0.01 M did not alter binding by >30% from that observed with buffer alone. It appeared, therefore, that there were no specific requirements for common cations in binding ligand to receptor. Also, heavy metallic cations did not appear to be required for binding since a number of chelating agents had little effect (table 4).

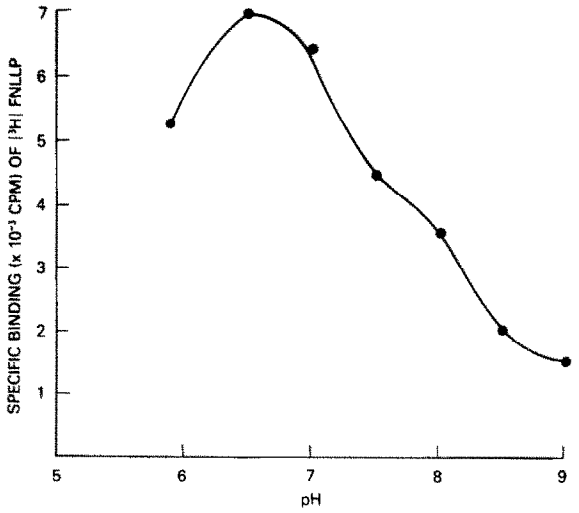

Fig.1. Variation of specific binding with pH. Assay conditions for specific binding were as in section 2, except that the buffer used in maintaining various pH values was Tris–acetate (0.1 M) and membrane preparations equivalent to 2.2 X 10^6 cells/ml were employed. The values are means of triplicate measurements which varied <10%.

3.5. *Effects of various treatments of whole cells and particulate preparations upon specific binding*

We attempted to obtain information on the chemical nature of the putative receptor in whole cells by subjecting them to a variety of pretreatments before they were assayed for their ability to bind ligand (table 4). Prior incubation with a variety of enzymes did not depress binding appreciably. In fact, some enzymes enhanced binding (papain by 49%; phospholipase A by 65%). This suggested that more receptors could be exposed by digesting away protein and small amounts of lipid constitutents of the cell membrane. In contrast, treatment with either 8 M urea or Triton X-100 eliminated binding of the ligand to whole cells and to particulate preparations. Results of using probes for roles of gangliosides and neuraminic acid residues in binding were negative as were tests for specific sugars as part of the binding site utilizing a variety of lectins in both whole cells and particulate preparations. Cell viability appeared unimpaired by treatment with these enzymes and reagents, except for 8 M urea and Triton X-100.

To identify functional groups (table 5) we used particulate preparations in order to eliminate internalization of receptor and attractant that might occur in whole cells. *p*-Mercuribenzoate (pMB) pretreatment at 10 μM caused >90% decrease in binding which was largely reversed by subsequent treatment with 0.1 mM dithiothreitol. The latter reagent caused only a 25% decrease in binding. These results suggest the participation of free sulfhydryl groups in binding. The effect of pMB appears to be dependent on temperature since the simultaneous incubation of pMB and fNLL[^{3}H]P with receptor-bearing fragments under assay conditions (0°C) did not affect binding. Amino-group reagents did not affect binding. From these results, which have been confirmed by Vitkauskas et al. (personal communication), it is not

Table 4
Effects of enzyme treatments and various reagents upon receptor-binding activity

A. Enzymatic treatment[a]	Percent change in specific binding	B. Reagents[b,c] added	Percent change in specific binding
Protease (100 μg/ml)	−2	Concanavalin A (200 μg/ml)	+6
Papain (100 μg/ml)	+49	Galactose-binding protein (200 μg/ml)	−8
Phospholipase A (250 μg/ml)	+65	Soy bean agglutinin (200 μg/ml)	−4
Phospholipase C (250 μg/ml)	−1	Fucose-binding protein (200 μg/ml)	+13
Phospholipase D (250 μg/ml)	−8	Ganglioside mixture (1 mM)	+9
Neuraminidase (100 μg/ml)	−8	Triton X-100 (1%)	−93
Glucosidase (100 μg/ml)	+10	Urea (8 M) (0.1 mM)	−90
Galactosidase (100 μg/ml)	+16	(0.1 M)	
Collagenase (100 μg/ml)	−20	EDTA (0.1 mM)	+4
Elastase (200 μg/ml)	0		
		α,α-Dipyridyl (0.1 mM)	+25
		Cupferron (0.1 mM)	−10

[a] Cells (2.2×10^{-6}/ml) were preincubated for 30 min at 37°C with the enzyme. The cells were washed free of added enzyme and suspended in Gey's solution and receptor binding assayed as in section 2

[b] Values are means of triplicate assays that varied <10%; 3 experiments were done

[c] Reagents were present only during the assay procedures, except in the cases of Triton and urea, which were removed before assay of binding

Table 5
Effect of sulfhydryl reagents upon specific binding of fNLL[^{3}H]P to particulate preparations of PMNs

Pretreatment[a]	Percent of control binding[c]
None	100
p-Mercuribenzoate, 10^{-5} M	8
p-Mercuribenzoate, 10^{-5} M followed by dithiothreitol, 10^{-4} M	70
Dithiothreitol, 10^{-4} M	75
p-Mercuribenzoate, 10^{-5} M under assay conditions[b]	96

[a] All pretreatments were done at 25°C for 40 min and the reagents removed before the binding assay

[b] The reagent was present only during the binding assay (1 h at 0°C)

[c] Values are means of triplicate assays that varied <10%; 4 experiments were done

possible to exclude any of the major classes (proteins, lipids, glycoconjugates) of membrane structural components as parts of the receptor.

4. Discussion

We have studied some properties of the putative receptor for formylated chemotactic peptides in rabbit neutrophils. We have found that the binding of peptides to this receptor correlates well with the cell's chemotactic responsiveness and lysosomal-enzyme releasing capacity. The ID_{50} values for a number of compounds are very close to their respective ED_{50} values for typical peptides over a 5 orders of magnitude range of activities, substantiating a physiological role for the receptor. There is a stereospecific preference that all amino acid residues possess the L-configuration. This applies to the antagonists as well as to the agonists. The antagonist with all L-residues has a binding affinity some 2 orders of magnitude less than the agonist with residues of the same configuration. However, the same antagonist binds as well as the diastereoisomeric agonist (f-L-Met–D-Leu–L-Phe). It is possible that most effective binding may require at least 3 sites of attachment to the receptor. The receptor appears to be a readily accessible surface component [5,12] which binds ligand with high affinity in a saturable, time-dependent process at low temperature. At higher temperatures (unpublished) we could not obtain saturable binding nor complete release of pre-bound ligand, suggesting that some internalization of ligand–receptor complex had occurred as has been indicated for epidermal growth factor [12] and shown recently in human neutrophils [7,14,15].

These properties (high affinity, structural specificity, steric selectivity) result in a highly sensitive and discriminating cellular response, possibly involving hydrolysis of the peptide attractant by the cell [11]. A role for hydrolysis might be to free the receptor from a molecule of attractant which had already delivered its signal, allowing the receptor to interact with a new molecule of attractant and sharpening the gradient in the vicinity of the cell [16]. There could, then, be two functionally distinct regions in the receptor, a ligand binding site and an hydrolytic enzyme. Support for this is the finding that the proteolytic inhibitor TPCK (but not TLCK), on one hand, inhibited chemotaxis and, on the other, increased binding to the receptor. A bound chymotrypsin-like esterase has been demonstrated on a neutrophil plasma membrane fraction also, which was the most enriched in chemotactic peptide binding sites of all the cellular subfractions [17]. However, the action of TPCK may be complex since it reacts with free sulfhydryl groups.

Free sulfhydryl groups may be required for ligand-receptor binding since pMB reversibly inhibits this binding. This may result from an interaction not directly at the binding site because pMB is not structurally related to chemotactic peptides. The sulfhydryl requirement, also found in the glucocorticoid receptor [18], is evidence in favor of the receptor containing an essential protein component.

The properties of the receptor appear to be consistent with the unique defensive role of the neutrophil as an inflammatory cell. It has high affinity for exceedingly small concentrations of the peptide attractant produced by *E. coli* [4] which is probably related to the formylated peptides used here. Therefore, the neutrophil can migrate along a gradient of attractant released by infecting bacteria. Chemotaxis in the presence of relatively low levels of attractant may require only a small proportion of receptor occupancy since the ED_{50} for chemotaxis is less than the ID_{50} for binding for a given peptide. Upon arriving at the site of infection (high levels of bacterial attractant), the neutrophil phagocytizes the bacteria, behavior involving a secretion of lysosomal enzymes. This is consistent with the properties of the receptor since the ED_{50} for lysosomal enzyme release is greater than that for chemotaxis. This may be an example of the same receptor modulating different cellular activities as a function of degree of receptor occupancy. Maximal binding occurs over the lower than physiological pH 6.5–7.0. Unlike other receptors, its binding activity, but not its internalization [7], appears both to be independent of the presence of a number of common cations and resistant to the action of proteolytic enzymes. It binds ligand appreciably over a broad range of temperatures. Upon its arrival at a site of infection, these properties would clearly be an advantage to the neutrophil, which must exercise its defensive function in a milieu of deranged homeostasis: low pH; variation in temperature; ionic imbalance; and high levels of proteolytic activity.

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