BINDING OF THE CHEMOTACTIC SYNTHETIC PEPTIDE [^{3}H]FORMYL–NOR-LEU–LEU–PHE TO PLASMA MEMBRANE OF RABBIT NEUTROPHILS

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1. Introduction

The chemotactic activity of a number of synthetic peptides has been described recently [1–3]. In addition to being chemotactic for neutrophils, they increase random migration, induce lysosomal enzyme release in the presence of cytochalasin B, and cause many other changes [3–8]. Recent reports have suggested that the primary interaction of these peptides with neutrophils leading to chemotaxis or lysosomal enzyme release is the binding of these compounds to a stereospecific receptor on the neutrophil surface [9,10]. This conclusion was based on the findings that these synthetic peptides bind specifically to intact rabbit and human neutrophils and their strength of binding is directly related to their biological activity [9,10]. In addition, the binding of the radiolabelled, chemotactic peptide, formyl–norleucyl–leucyl–phenylalanine, was found to be in the membrane fraction of rabbit neutrophils [11], providing additional support to the hypothesis that the receptors for these synthetic peptides are, indeed, located on the cell surface. However, in this latter investigation no attempt was made to study the binding characteristics of the membrane to the peptide or to test whether there was any change in the nature of the binding of the peptide to the isolated membrane compared to whole cells.

The present studies were undertaken to test directly whether any change in the binding site for [^{3}H] F–N-Leu–Leu–Phe occurred consequent to the isolation of the plasma membrane. This is necessary information since as planned, the membrane fraction is to be used as a source for the further purification of the receptor. Using the radiolabelled peptide [^{3}H], F–NLeu–Leu–Phe a potent chemotactic agent, we have investigated the binding characteristics of both whole cells and plasma membrane fractions of rabbit neutrophils. Binding to either whole cells or plasma membranes is an extremely rapid and saturable process. The equilibrium dissociation constant, K_d, for either the whole cell or for the membrane fraction was found to be in $(2.7 \pm 0.5) \times 10^{-8}$ M and $(2.9 \pm 0.3) \times 10^{-9}$ M, respectively.

2. Materials and methods

Polymorphonuclear leukocytes were obtained from white albino rabbits by injection of 400 ml of 0.1% glycogen in sterile saline and collecting the leukocyte rich exudate 12 h after injection. The suspension was gently centrifuged at ($250 \times g$) for 5 min. The supernatant was removed and the packed cells were resuspended in 200 ml of isotonic buffered NH_4Cl. This procedure was necessary to hemolyze red cells [5,12,13]. The suspension was then centrifuged for 5 min at $250 \times g$. The supernatant was aspirated and the pellet was gently resuspended in 200 ml of 11.6% sucrose, 10 mM Tris–HCl, pH 7.4 and spun at ($250 \times g$) for 5 min. The pellet was then gently resuspended in the 11.6% sucrose solution containing 1 mM EDTA as well as 10 mM Tris–HCl, pH 7.4, and spun again for 3 min at ($250 \times g$). The pellet was homogenized in a T-Line Laboratory Homogenizer (Talboys Engineering Corp., Emerson, N.J.) for 2 min at approximately 1000 rev./min until all the cells were broken as determined by microscopic examination.

Membranes were prepared from the homogenate by a slight modification of the method of Woodin and Wieneke [13–15]. In this method, the postnuclear fraction was obtained by centrifugation of the above described cell homogenate at 1000 × *g* for 10 min. The supernate was layered on a discontinuous gradient containing 3 ml of 30% (weight by volume) sucrose, 3 ml of 40% sucrose and 3 ml of 50% sucrose. All of the sucrose solutions contained 1 mM EDTA and 10 mM Tris–HCl buffer, pH 7.4. The tubes were placed in a SW40 rotor and the gradients were centrifuged at 111 681 × *g* for 1 h at 4°C. Band I was formed between the suspending medium and 30% sucrose, band II between the 30% and 40% sucrose, and band III between the 40% and 50% sucrose. The material designated as band II is referred to as plasma membrane and that designated as band III is the cytoplasmic membrane fraction [13–15]. The bands were transferred to clean centrifuge tubes and diluted with cold 16.4 mM Tris-HCl, pH 7.6 and centrifuged at 105 536 × *g* for 1 h. The pellets were then resuspended in 16.4 mM Tris solution. Protein concentration was determined by the method of Lowry et al. [16] using bovine serum albumin as standard. We have shown previously that only band II contains an ATPase activity [13,14] which meets all the requirements (Na^+-activated, K^+-activated and ouabain inhibited) of the commonly known 'Na^+, K^+'-ATPase involved in the maintenance of the Na^+ and K^+ concentration gradients across all plasma membranes.

The synthetic peptide [^{3}H] F–NLeu–Leu–Phe was synthesized and labelled as previously described [1,17]. Binding was carried out by a filtration technique using a constant amount of radioactivity and varying concentrations of the cold synthetic peptide. The incubation was begun by the addition of 0.1 ml of neutrophil membrane to 0.1 ml of a peptide solution containing a known amount of both labelled and nonlabelled peptide in Hank's buffer. It was terminated by the rapid dilution of this mixture with 3 ml of ice–cold Hank's buffer followed by filtration of the mixture through a Whatman GF/C glass fiber filter attached to a low pressure chamber. The Hank's buffer contained mM concentration: NaCl, 124; KCl, 4.86; $NaHCO_3$, 15.1; Na_2CO_3, 0.63; KH_2PO_4, 0.66; Hepes buffer, 10 mM; pH 7.3. Each tube was subsequently rapidly washed twice with a total of 6 ml of ice-cold Hanks', after which the filters were washed with another 3 ml of the cold incubation buffer. The filters were then dried and placed into vials containing 12 ml of the scintillation fluid, Aquasol (NEN, Boston, Mass.). Radioactivity was measured in a liquid scintillation spectrophotometer.

Nonspecific binding was defined as the amount of binding not inhibited by 2×10^{-5} M unlabelled Formyl–NLeu–Leu–Phe. Specific binding was defined as the total amount of [^{3}H] F–NLeu–Leu–Phe bound minus the nonspecific binding. It should be noted that the values of binding in all figures and tables refer to specific binding.

3. Results and discussion

The time course of the specific binding of the synthetic peptide [^{3}H] F–NLeu–Leu–Phe to both plasma membrane (band II) and whole cells of rabbit neutrophils are shown in figs.1A and 1B, respectively. As is evident from the figure, the specific binding to both the membrane fraction and whole cells is rapid with a half-time of less than 1 min at 25°C, and is completed with the first 5 min. The rate of binding is much more rapid than reported by Aswanikumar et al. [9] but is in agreement with unpublished indepen-

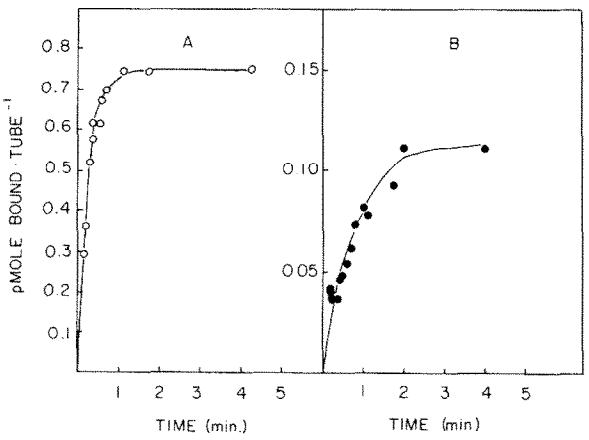

Fig.1. Time course of the synthetic peptide [^{3}H]–Formyl–NLeu–Leu–Phe specific binding to plasma membrane (A) and whole cells (B) from rabbit neutrophils at 25°C. In the case of membranes each tube contains 0.76×10^6 cells. The total concentration (labelled + unlabelled) of the peptide is 70 nM.

dent findings of Dr. G. Vitkaushes in the laboratory of one of us (E.L.B.) and also with the rate of enzyme release. Small amount of specific binding of this synthetic peptide was observed in band I which is probably due to contamination by plasma membrane. No binding was observed in band III.

In order to determine the affinity and the number of binding sites to both whole cells and plasma membranes, experiments were designed for Scatchard plot analysis. In these experiments, the range of peptide concentration (labelled + unlabelled) used was between 1.09 and 104 nM. In all of these experiments, the reactions were incubated at 25°C and terminated after 5 min. A typical Scatchard plot of the specific binding of [^{3}H] F–NLeu–Leu–Phe to the plasma membrane (band II) isolated from rabbit neutrophils is shown in fig.2. The results of Scatchard plot analysis for various experiments dealing with the specific binding of the peptide to the plasma membrane are shown in table 1. The mean value of $2.9 \pm 0.3 \times 10^{-9}$ M for the equilibrium dissociation constant, K_d, is close

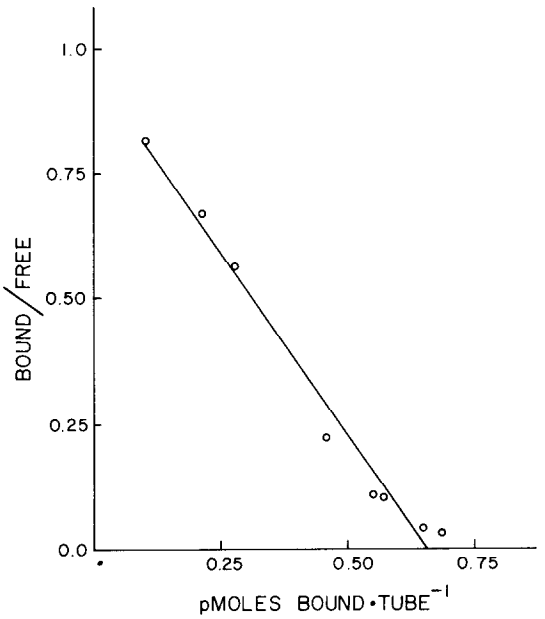

Fig.2. Scatchard plot of the synthetic peptide [^{3}H]Formyl–NLeu–Leu–Phe specific binding to plasma membrane obtained from rabbit neutrophils at 25°C. Each tube contains 20 μg protein. The range of concentration of the total (labelled + unlabelled) peptide used was between 1.09 nM and 104 nM. The reactions were terminated after 5 min of incubation. The dissociation constant, K_d, and number of receptor binding sites, R_o, were calculated using the relationship

$$\frac{\text{Bound}}{\text{Free}} = -\frac{1}{K_d}(\text{bound}) + \frac{R_d}{K_d}$$

where 'free' is the total amount of peptide added minus the amount bound.

Table 1
Parameters of Scatchard plot analysis of the specific bindings of [^{3}H]Formyl–NLeu–Leu–Phe to the plasma membrane (Band II) isolated from rabbit neutrophils at 25°C

Exp.	Equilibrium dissociation constant (K_d) in molar	Number of binding sites (R_o) sites/mg protein
1	2.9×10^{-9}	7.4×10^{12}
2	1.5×10^{-9}	6.4×10^{12}
3	2.8×10^{-9}	10.7×10^{12}
4	2.0×10^{-9}	8.5×10^{12}
5	3.4×10^{-9}	8.5×10^{12}
6	3.2×10^{-9}	6.5×10^{12}
7	3.7×10^{-9}	10.1×10^{12}
8	1.2×10^{-9}	10.0×10^{12}
9	5.4×10^{-9}	8.3×10^{12}
10	2.4×10^{-9}	16.6×10^{12}
Mean ± SEM	$2.9 \pm 0.3 \times 10^{-9}$	$9.3 \pm 0.9 \times 10^{12}$

Each Scatchard plot straight line has a correlation coefficient of greater than 0.96

Table 2
Parameters of Scatchard plot analysis of the specific bindings of [^{3}H]Formyl–NLeu–Leu–Phe to the intact cell of rabbit neutrophils at 25°C

Equilibrium dissociation constant (K_d) in molar	Number of binding sites (R_o) sites/cell
$2.7 \pm 0.5 \times 10^{-8}$ (6)	$76 \pm 10 \times 10^3$ (6)[a]

[a] The values represent the means and the standard error of the means of 6 different experiments

Each Scatchard plot straight line has a correlation coefficient of greater than 0.95

to the concentration of the synthetic peptide required to elicit a half-maximal chemotactic response, 7×10^{-10} M [1]. The results of Scatchard plot analysis for various experiments dealing with the specific binding of the peptide to the whole cells are summarized in table 2. The mean value of $2.7 \pm 0.5 \times 10^{-8}$ M for the dissociation constant, K_d is distinctly higher than the value of 1.5×10^{-9} M reported Aswanikumar [9] for the same cells. On the other hand, the mean value of 76 000 ± 10 000 for the number of binding sites per cell calculated assuming an equimolar ligand–receptor complex is essentially the same as the value of 10^5 sites reported by Aswanikumar et al. [9]. These differences are likely due to the different experimental conditions.

Because of a peptidase present on neutrophils which hydrolyzes and inactivates the chemotactic peptides [18], Aswanikumar et al. [9] pretreated their neutrophils with an inhibitor of the peptidase, tolyl-*L*-phenylalanine chloromethyl ketone (TPCK) and did their binding at 4°C. We did not pretreat the cells with TPCK and carried out the binding at room temperature. Thus our finding of a higher value for K_d of binding but the same number of sites as Aswanikumar et al. [9] is explicable, if under our conditions, the neutrophil peptidase inactivates a fraction of the peptide presented to the cell. The fact that the K_d of binding of the membrane fraction, $2.9 \pm 0.3 \times 10^{-9}$ M is essentially the same as that found by Aswanikumar et al. [9] for the whole cell suggests that the peptidase is either not on the plasma membrane or its activity is largely lost in the preparation of the membrane. In any event the results obtained support the conclusion that the preparation of the membrane fraction does not significantly change the binding properties of the peptide receptor.

Using the mean value of $(4.3 \pm 0.2) \times 10^{-8}$ mg protein/cell determined from 15 different experiments, the 76×10^3 sites/cell is equivalent to 1.77×10^{12} sites/mg protein comparing this with the value of 9.3×10^{12} sites/mg protein obtained using the membrane preparation indicates that there is at least a 5-fold increase in the specific activity of these receptors.

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