Characterization of the Rat Neutrophil Formyl Peptide Chemotaxis Receptor

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Numerous synthetic N-formylated peptides, believed to be the analogs of the naturally occurring initiating signal peptides produced by bacteria, are potent chemotactic agents for phagocytic cells in several species. The authors have characterized the receptor with moderately high affinity for the chemotactic peptide f-Met-Leu-[^{3}H]Phe on the rat peritoneal neutrophils. When neutrophils are incubated with f-Met-Leu-[^{3}H]Phe at 24 C, the binding is saturable and reversible. The receptor on the inflammatory rat neutrophils has an equilibrium dissociation constant (K_D) of 3.4 × 10^{-8} M at 24 C, and there are approximately 65,000 sites per cell. In addition, the potency of several of these chemotactic peptides in inducing lysosomal enzyme secretion and superoxide production correlated well with their ability to compete with f-Met-Leu-[^{3}H]Phe for receptor binding. Structure activity studies further demonstrate that the fine specificity of the formyl peptide receptor has been conserved across species lines. (Am J Pathol 1983, 111:273–281)

THE ACCUMULATION of acute inflammatory cells at sites of bacterial infection is believed to be mediated, at least in part, through the binding of bacterial signal peptides to a specific cell surface receptor on the neutrophil and macrophage.[1] This receptor can bind numerous synthetic formylated oligopeptides of two to six amino acids in length and subsequently shows varying degrees of biologic reactivity. Structure activity studies with rabbit neutrophils have demonstrated that specificity for binding to the receptor depends not only on the amino acids present in the formylated peptides, but also on their relative position to one another in the polypeptide chain.[2–4] These studies as well as others which use reagents that modify specific amino acids on the cell surface have led to a hypothesis of the structure of the active site of the formyl peptide receptor.[4–5]

The formyl peptide receptor has been demonstrated on polymorphonuclear leukocytes (PMNs), macrophages, and other mature cells of the myeloid series[6–7] and in several species, including rabbit, human, guinea pig, horse, mouse, and rat.[8–14] With the last, a thorough analysis of the nature and specificity of the receptor has not been reported. We therefore undertook this study to characterize in detail the formyl peptide receptor of the rat neutrophil.

Materials and Methods

Materials

Oligopeptides

The synthetic peptides used in this study were 2-ethyl-hexanoyl-Leu-Phe, f-Ala-Leu-Phe, f-Abu-Leu-Phe (Abu, α-aminobutyric acid), f-Met-Leu-Glu, f-Met-Abu-Phe, f-Met-Cys(Me)-Phe (Cys[me], methyl cysteine), f-Nle-Leu-Phe (Nle, norleucine), f-Met-Leu-Phe-BzA (BzA, benzylamide), f-Met-Leu-Phe-Phe, f-Met-Leu-Phe, f-Met-Gly-Phe, Met-Leu-Phe, f-Met-Leu-Phe-α-Lys, and f-Met-Leu-Phe-ε-Lys. The synthesis and biologic activity of these peptides for rabbit neutrophils have been reported elsewhere.[2–4] The radiolabeled peptide, f-Met-Leu-[^{3}H]Phe (47.6 Ci/mmol), and ^{3}H-sucrose (11.2

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Ci/mmol) were obtained from New England Nuclear, Boston, Massachusetts. Sodium azide, 2-deoxyglucose, oyster glycogen, crystalline bovine serum albumin, ferricytochrome C, superoxide dismutase, formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe), and *p*-nitrophenyl-N-acetyl-β-D-glucosaminide were purchased from Sigma Chemical Co., St. Louis, Missouri. Cytochalasin B was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Boc-Phe-Leu-Phe-Leu-Phe (Boc-tert-butoxy) was obtained through Peninsula Laboratories, Inc., San Carlos, California.

Methods

Cells

Retired breeder Sprague–Dawley rats were injected with 30 ml sterile 0.1% oyster glycogen in saline and sacrificed 18 hours later with ether. The peritoneal cavities were opened surgically and the cells obtained by repeated lavage with Hanks' medium containing 10 units/ml sodium heparin. For all experiments, cells from 3 or 4 rats were pooled. Morphologic analysis showed greater than 85% polymorphonuclear leukocytes as determined by differential Wright's stain.

Measurement of f Met-Leu-[^{3}H]Phe Binding

All binding studies were carried out at 24 C in Hanks' medium supplemented with 1.6 mM calcium, 10 mM sodium azide and 10 mM 2-deoxyglucose at a cell concentration of 2–4 × 10^7 cells/ml. Cells were always preincubated at 24 C for 30 minutes before initiation of a binding study at the same temperature. The binding studies were performed by incubating 100 μl of cells and 5 μl of radiolabeled peptide in a 12 × 75-mm glass test tube. After 20 minutes (unless otherwise specified), the cells were harvested by a glass fiber vacuum filtration method[15,18] and analyzed for cell-bound radioactivity. Nonspecific binding was defined as the amount of f-Met-Leu-[^{3}H]Phe bound in the presence of a 1000-fold molar excess of nonradiolabeled f-Met-Leu-Phe and was always less than 10% of total binding. Throughout this article, "specific binding" refers to the total binding minus nonspecific binding. All data points were obtained in duplicate or triplicate, and the standard error of the mean was consistently less than ± 5%.

For dissociation experiments, the reversibility of f-Met-Leu-[^{3}H]Phe binding after incubation for varying times was determined by the addition of a 1000-fold molar excess of unlabeled f-Met-Leu-Phe. At various times thereafter, cells were harvested and assayed for loss of cell-bound peptide. In some cases the reversibility of binding was also measured by varying the time of the wash with cold (4 C) buffer.[16] All data points were obtained in duplicate.

The specificity of the formyl peptide receptor was determined by inhibition of f-Met-Leu-[^{3}H]Phe binding. In brief, PMNs and 0.6–1.0 pmol f-Met-Leu-[^{3}H]Phe were incubated for 20 minutes in the presence of 5–10-fold dilutions of the various oligopeptides. Specific binding was calculated and is expressed as the percentage of inhibition of f-Met-Leu-[^{3}H]Phe binding.

Pinocytosis

The fluid phase uptake of ^{3}H-sucrose at 24 C was investigated in medium containing 10 mM glucose alone or 10 mM 2-deoxy-glucose and 10 mM NaN_3. Cells were incubated with 10^{-6} M ^{3}H-sucrose in the presence or absence of 10^{-6} M f-Met-Leu-Phe. After various times of incubation, the cells were resuspended in 10 ml of 4 C Hanks' medium for 5 minutes. They were then washed, and the cell associated radioactivity was assayed as previously described.

Biologic Studies

Lysosomal enzyme release was performed at 5 × 10^6 cells/ml in the presence of 5 μg cytochalasin B and was terminated after 5 minutes at 37 C by immersing the tubes in an ice bath, followed by centrifugation. N-acetyl-β-D-glucosaminidase was assayed in the supernatant by measuring the release of p-nitrophenyl from its substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide.[17] Lysozyme was assayed by measuring turbidometrically the lysis of *Micrococcus lysodeikticus*.[2]

To assay the peptides' respective abilities to induce O_2^- production, we incubated rat neutrophils in Hanks' buffer for 10 minutes at 37 C, and cytochalasin B was added (5 μg/ml). Then 400 μl of cells (5 × 10^6 cells/ml) were dispensed into 12 × 75-mm glass test tubes to which had been added 25 μl of either Hanks' buffer or a 1-mg/ml solution of superoxide dismutase (SOD), 200 μl of a 0.23 mM solution of ferricytochrome C, and 50 μl of the appropriate dilution of peptide. The mixtures were then incubated at 37 C for 30 minutes, and the reaction was stopped by the addition of 425 μl of Hanks' buffer to tubes that had previously received 25 μl of SOD and 400 μl of Hanks' buffer plus 25 μl of SOD to tubes that did not contain SOD. The tubes were centrifuged at 4 C at 700*g* for 5 minutes, and the absorbance of the supernatant was read at 550 nm with a Varian Series 634 spectrophotometer (Varian Techtron Inc., Park Ridge, Ill). The amount of O_2^- produced was calculated from the difference in ab-

sorbance of the samples with and without superoxide dismutase. This difference was divided by the extinction coefficient for the change between ferricytochrome C and ferrocytochrome C.

Results

Time Course for f-Met-Leu-[^{3}H]Phe Binding

The specific binding of f-Met-Leu-[^{3}H]Phe (6.25 nM) to rat neutrophils was investigated at 24 C, (Figure 1). Maximal binding at 24 C was reached by 20 minutes and was maintained up to 60 minutes; however, after this time, a steady decline in cell associated peptide was seen. At 2 hours, the loss of cell-bound peptide at 24 C had stabilized, and a new plateau was reached. Nonspecific binding was always less than 10% of specific binding even after 4 hours of incubation.

Binding of f-Met-Leu-[^{3}H]Phe at Equilibrium

Because maximum receptor occupancy at 24 C was stable for a sufficient period of time to permit an accurate determination of binding characteristics, all subsequent experiments were performed under these conditions.

The effect of f-Met-Leu-[^{3}H]Phe concentration on specific binding and receptor saturability is shown in Figure 2. Saturation of formyl peptide receptors does occur at high concentrations of f-Met-Leu-[^{3}H]Phe

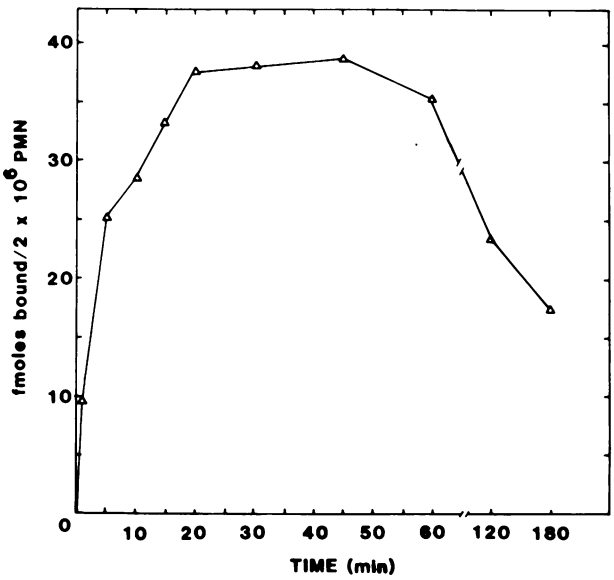

Figure 1—Time course on f-Met-Leu-[^{3}H]Phe binding to rat PMNs; 2 × 10^6 PMNs were incubated at 24 C for various time intervals, as indicated with 6.25 nM f-Met-Leu-[^{3}H]Phe. Nonspecific binding was determined in the presence of 6.25 μM unlabeled f-Met-Leu-Phe.

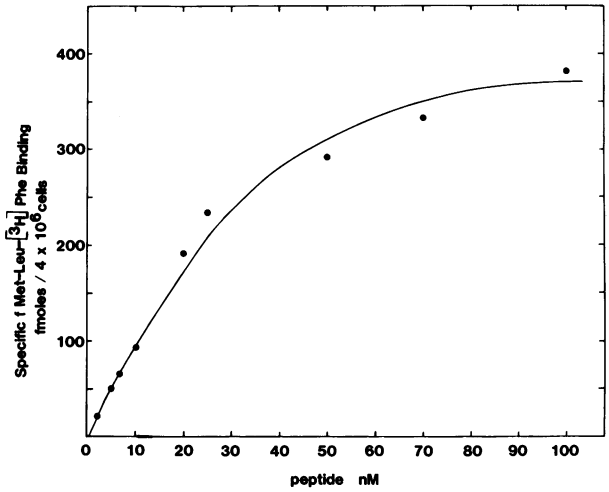

Figure 2—Specific binding of f-Met-Leu-[^{3}H]Phe to inflammatory PMNs as a function of f-Met-Leu-[^{3}H]Phe concentration. F-Met-Leu-[^{3}H]Phe was incubated at the given concentration for 20 minutes at 24 C with 4 × 10^6 cells, and the specific binding was calculated. Each point is the mean of triplicate determinations.

(ie, 80 nm). When specific binding is determined for each concentration of peptide and analyzed according to the method of Scatchard,[19] a linear relationship is obtained (Figure 3). This demonstrates that rat neutrophils have a single class of noninteracting receptors for the formyl peptides with a dissociation constant (K_D) = 3.45 ± 1.18 × 10^{-8} M (mean ± SEM for 4 experiments), and there are 63,629 ± 11,479 binding sites per cell (range 36,000–82,000). This is similar to the known affinity and number of receptors per cell found on rabbit and human neutrophils[8,20]; but the number of receptors appears distinctly higher than found on horse PMNs,[12] guinea pig macrophages,[11,12] or human monocytes.[22]

Dissociation of Cell-Bound f-Met-Leu-[^{3}H]Phe

To ensure that the dissociation constant (K_D) calculated by Scatchard analysis was done under equilibrium conditions, the reversibility of binding of f-Met-Leu-[^{3}H]Phe to its receptor was investigated. Figure 4 shows that at 24 C, the addition of a 1000-fold excess of unlabeled peptide caused the loss of cell-bound peptide. Similar results were obtained by varying the time of the wash with cold (4 C) buffer. This reversibility of binding is evident at 1, 5, and 15 minutes, however, after longer periods of incubation—30 minutes—a significant decrease in the amount of dissociable cell-bound peptide was observed, even though 10 mM NaN_3 and 10 mM deoxyglucose were included in the incubation medium. After 1 and 5 minutes of incubation with f-Met-Leu-[^{3}H]Phe, approximately 50–70% of the

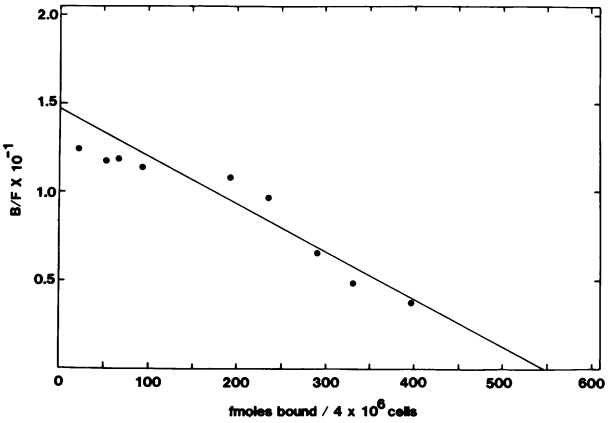

Figure 3—Scatchard analysis of specific f-Met-Leu-[^{3}H]Phe binding to rat PMNs. Free ligand is the amount of f-Met-Leu-[^{3}H]Phe added minus the total f-Met-Leu-[^{3}H]Phe binding. The equilibrium dissociation constant (K_D) and the number of binding sites per cell were calculated from the slope and *x*-intercept, respectively. (r = 0.96).

specifically bound radioactivity was dissociated during a 15-minute incubation of cells with unlabeled peptide. However, after an initial 15-minute incubation with f-Met-Leu-[^{3}H]Phe, only 30% (range 20–40%) of the specifically bound radioactivity was dissociated following a 15-minute incubation with unlabeled peptide, whereas after an initial 30-minute incubation with f-Met-Leu-[^{3}H]Phe only approximately 20% (range 10–30%) of the specifically bound peptide was dissociated. Thus, after binding of the peptide to its receptor, a time-dependent process occurs that alters the receptor–peptide complex such that dissociation is no longer possible.

The rate constant for dissociation (k_{off}) at 24 C was calculated with the use of the 5-minute incubation shown in Figure 4. As can be seen in Figure 5, the rate constant for dissociation of cell-bound peptide was 0.047 minutes^{-1} (mean 0.066 ± 0.008 min^{-1} [n = 7]; range 0.044–0.10 minutes^{-1}). The t½ for peptide dissociation at 24 C was 6.98 ± 0.83 minutes (n = 7). These values for the dissociation rate constant at 24 C are greater than the value obtained by analyzing the rate of approach to equilibrium binding where k_{off} = 0.033 min^{-1} (Figure 5) (see below). The significance of this twofold difference for the half-time of dissociation as determined by the two methods is unclear. Whether this is evidence of either the presence of cooperative interactions[23] or the existence of more than a single class of independent receptor sites[24,25] is under investigation.

Kinetic Analysis of the Binding of f-Met-Leu-[^{3}H]Phe

The binding of f-Met-Leu-[^{3}H]Phe to rat PMNs at 24 C is rapid. In the absence of irreversible processes (ie, internalization, degradation) the reversible interaction of peptide (P) and receptor (R) to form a peptide-receptor complex (PR) can be described by the reaction:

$$P + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PR \qquad (1)$$

By determining the initial rate (v_i) of the binding reaction, the rate constant for the formation of the peptide–receptor complex (k_{on}) can be calculated according to the equation

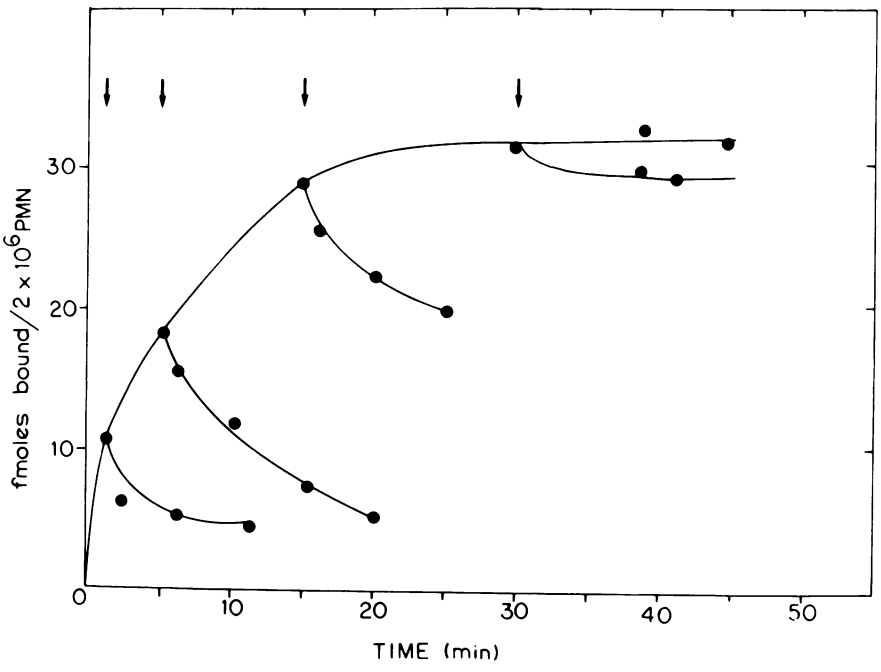

Figure 4—Reversibility of f-Met-Leu-[^{3}H]Phe binding at 24 C after incubation for varying times with 6.25 nM f-Met-Leu-[^{3}H]Phe. *Arrows* mark the point at which 6.25 μM unlabeled peptide was added to cells. At various time points thereafter, an aliquot of cells was removed, washed, and rapidly filtered.

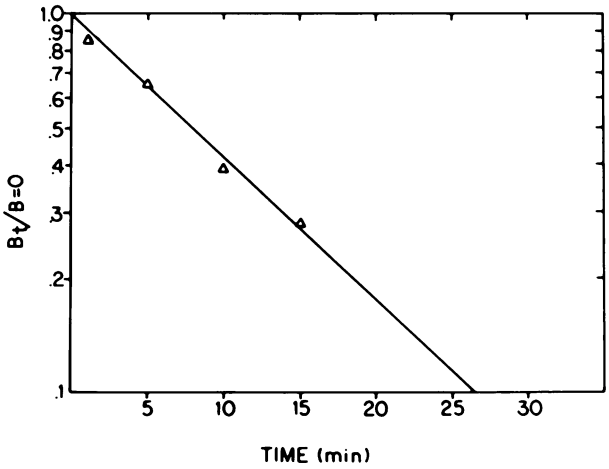

Figure 5— Rate of dissociation of f-Met-Leu-[^{3}H]Phe after binding for 5 minutes, ie, the reaction had proceeded to 60% of equilibrium. Rat PMNs (2×10^6 cells) were incubated with 6.25 nM f-Met-Leu-[^{3}H]Phe for 5 minutes. ($B = 0$) and the rate of dissociation of f-Met-Leu-[^{3}H]Phe binding was determined at various time points after the addition of 6.25 μM unlabeled f-Met-Leu-Phe. The amount of f-Met-Leu-[^{3}H]Phe remaining specifically bound to cells at different times (B_t) was determined as described in Methods. Each point represents the mean of duplicate determinations. The half-time for dissociation was 7.8 minutes, which corresponds to a rate constant $k_{off} = 0.047$ minutes^{-1}.

$$v_i = k_{on} (R_o) (P_o) \quad (2)$$

where (R_o) and (P_o) are the concentrations of receptor and peptide initially present. Using $R_o = 65{,}000$ sites/cell as determined by Scatchard analysis (Figure 3), the K_{on} is calculated to be $3.07 \pm 0.62 \times 10^6$ M^{-1} minutes^{-1} (mean ± SEM) (n = 3) for the first 5 minutes of the reaction shown as in Figure 1.

The rate of approach to equilibrium binding (k_{obs}) at 24 C was determined by plotting the specific binding from Figure 1 semilogarithmically as a function of time. A linear plot was obtained (data not shown) that indicates that the reaction followed pseudo-first-order kinetics even though the initial concentrations of tracer (6.25×10^{-9} M) and receptor (2.57×10^{-9} M) were of the same order of magnitude. In addition, since the concentration of free peptide remains essentially constant (ie, less than 5% of the total f-Met-Leu-[^{3}H]Phe was bound), the reaction kinetics are only dependent on the number of receptors available.[16,26] The half-time for approach to equilibrium was 6 minutes, which corresponds to a pseudo-first-order rate constant (k_{obs}) of 0.053 minutes^{-1}.

The rate of approach to equilibrium binding, k_{obs}, can also be used to derive the on and off rates.[26] The half-time for the approach to equilibrium is determined for various concentrations of peptide by plotting the fraction of equilibrium binding as a function of time

$$k_{obs} = \frac{\ln 2}{t½}$$

where t½ is the time at which PR reached one-half its equilibrium value. The rate of approach to equilibrium is described by the equation

$$\frac{d(PR)}{dt} = k_{on} (R) (P) - k_{off} (PR) \quad (3)$$

Under pseudo-first-order conditions, integration of this equation can be used to determine the k_{obs} at different concentrations of peptide:

$$k_{obs} = k_{on} (P) + k_{off} \quad (4).$$

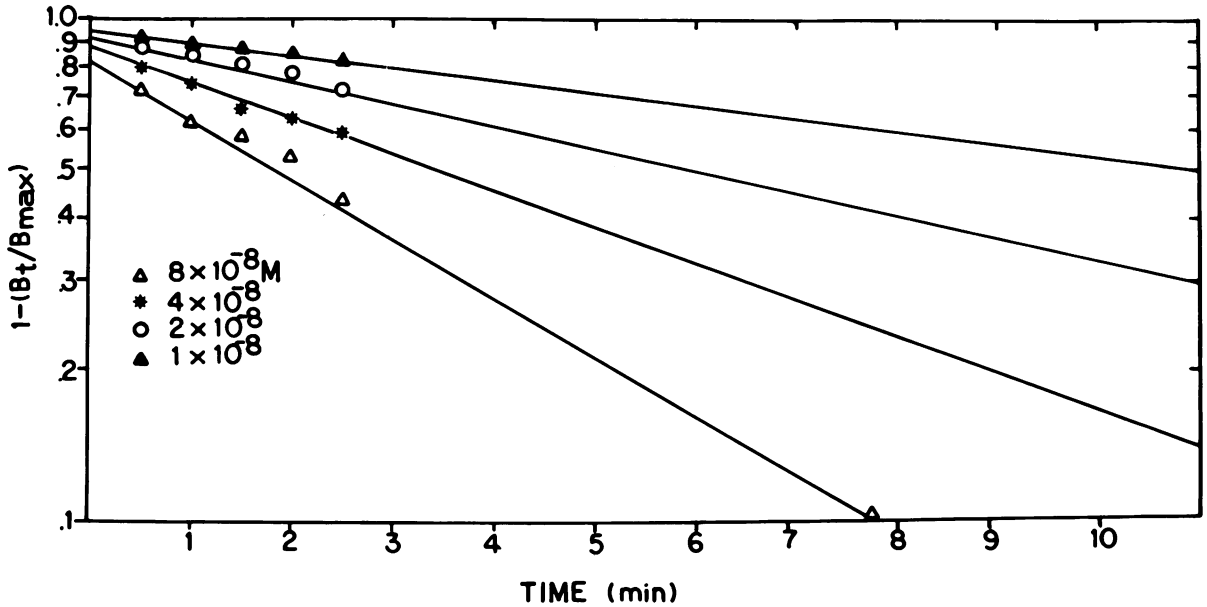

Figure 6— Rate of saturable f-Met-Leu-[^{3}H]Phe binding plotted semilogarithmically as a function of time. B_t, saturable binding at time t. B_{max}, saturable binding at equilibrium. Saturable binding is the difference in the amount of f-Met-Leu-[^{3}H]Phe binding in the absence and presence of 1000-fold excess unlabeled f-Met-Leu-Phe. △, 8×10^{-8} M: *, 4×10^{-8} M; O, 2×10^{-8} M; and ▲, 1×10^{-8} M f-Met-Leu-[^{3}H]Phe. The half-time values, $t_{½}$, for the approach to equilibrium binding are 1.9 minutes, 3.41 minutes, 6.05 minutes, and 10.45 minutes, respectively.

Figure 6 gives semilogarithmic plots of the rates of specific binding at four different peptide concentrations. The rate constants for approach to equilibrium determined from the data in Figure 6 were plotted as a function of the total peptide concentration according to equation 4 (Figure 7).

The rate constants calculated from the slope and intercept of this plot were $k_{on} = 6.28 \times 10^6$ M^{-1} $minutes^{-1}$, $k_{off} = 0.03$ $minutes^{-1}$. This value for k_{on} is twofold greater than the value obtained from the initial rate of f-Met-Leu-[^{3}H]Phe binding ($k_{on} = 3.07 \times 10^6$ M^{-1} $minutes^{-1}$). As a result of these 2 twofold differences in both of the kinetically derived rate constants (k_{on} and k_{off}), as compared with the on and off rates derived from the rate of approach to equilibrium binding, the kinetically derived $K_D = (k_{off}/k_{on})$ 5.25×10^{-9} M is of higher affinity than the dissociation constant obtained from equilibrium analysis, $K_D = 3.45 \pm 1.18 \times 10^{-8}$ M.

Specificity of the Formyl Peptide Receptor

The ability of various N-formylated oligopeptides to compete with f-Met-Leu-[^{3}H]Phe for binding to the rat neutrophil is shown in Table 1. A greater than 10,000-fold range of binding activities is seen for peptides modified in one or another of the proposed first five critical areas of peptide-receptor interaction.[4,5] In addition, strong similarities exist to the rabbit neutrophil in the specificity of its formyl peptide receptor.[2-5] In particular, removal of the formyl group, as in Met-Leu-Phe, decreased binding 800-fold as compared with f-Met-Leu-Phe. 2-Ethylhexanoyl-Leu-Phe, an aliphatic analog that resembles in its steric bulk the formyl-methionyl moiety, is also decreased in its binding 800-fold. Thus, as demonstrated across species lines, these data suggest that there is some unique property of the formyl group responsible for its dramatic effect.[4,9,11,12]

Our data also confirm the importance of methionine in Position 1. An approximate 10-fold decrease in binding affinity is seen with the isosteric aliphatic analog norleucine in f-Nle-Leu-Phe. This same effect is seen with rabbit and human neutrophils[3,9] and guinea pig macrophages[11] and supports the presence of a discrete area of positive charge in the hydrophobic pocket of the receptor that accommodates the methionine residue.[4,5]

Of the four amino acids that varied in Position 2 of the tripeptide, the aliphatic straight chain α-aminobutyric acid derivative has approximately the same activity as does the gamma-branched leucine when in Position 2, thus abrogating the requirement of gamma-branching in this position. In addition, the sulfur-containing straight chain derivative methyl cysteine is 40-fold less active than leucine in Position 2, whereas the uncharged polar amino acid glycine, in f-Met-Gly-Phe, is 3600 times less active than f-Met-Leu-Phe.

In Position 3, the phenylalanine residue has been shown to increase biologic activity dramatically over other nonpolar and polar amino acids.[2-5] With the rat neutrophil, we find the same results. A greater than 60-fold decrease in binding activity is seen when one replaces the nonpolar group phenylalanine with the negatively charged polar amino acid glutamic acid. These data are compatible with the receptor model of the rabbit neutrophil formyl peptide receptor and suggest that the hydrophobicity and/or aromaticity of phenylalanine is responsible for its profound effects.

In Position 4, contrasting effects are seen when the nonpolar phenylalanine versus the positively charged

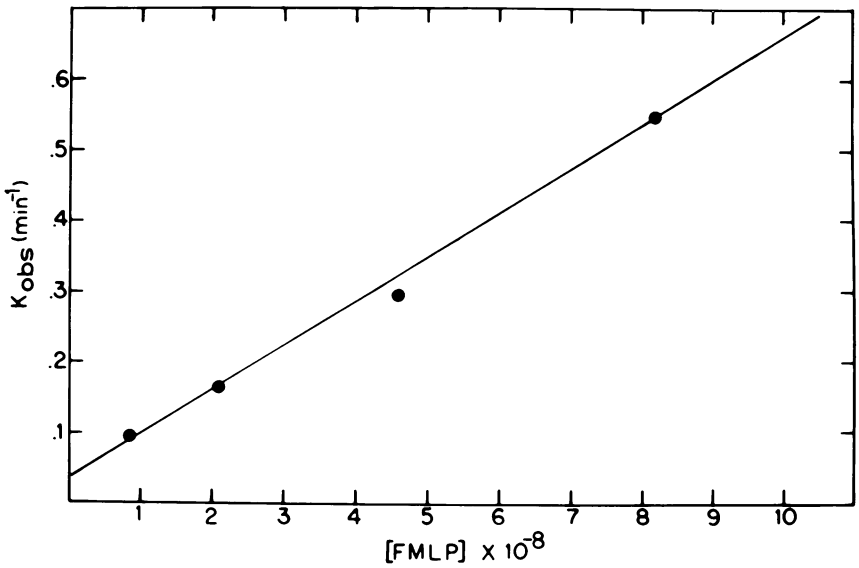

Figure 7—Rate of f-Met-Leu-[^{3}H]Phe binding, k_{obs}, calculated from the half-time to equilibrium binding in Figure 6 as a function of peptide concentration. The rate constants calculated from the slope and x-intercept are $k_{on} = 6.28 \times 10^6$ M^{-1} $minutes^{-1}$ and $k_{off} = 0.033$ $minutes^{-1}$, respectively.

Table 1—Comparison of the Displacement of Specific f-Met-Leu-[^{3}H]Phe Binding by Various Synthetic Oligopeptides

Peptide	Inhibition of f-Met-Leu-[^{3}H]Phe binding (ID_{50})*
f-Met-Leu-Phe	3.6×10^{-8}
Met-Leu-Phe	2.9×10^{-5}
2-Ethyl-hexanoyl-Leu-Phe	2.1×10^{-5}
f-Ala-Leu-Phe	ND†
f-Abu-Leu-Phe	ND
f-Nle-Leu-Phe	3.4×10^{-7}
f-Met-Gly-Phe	1.0×10^{-5}
f-Met-Abu-Phe	3.1×10^{-8}
f-Met-Cys(Me)-Phe	1.4×10^{-6}
f-Met-Leu-Glu	2.4×10^{-6}
f-Met-Leu-Phe-Phe	1.2×10^{-9}
f-Met-Leu-Phe-α-Lys	1.0×10^{-7}
f-Met-Leu-Phe-ε-Lys	3.8×10^{-7}
Boc-Phe-Leu-Phe-Leu-Phe	2.3×10^{-6}

* ID_{50} is the concentration of peptide required to displace 50% of the specific f-Met-Leu-[^{3}H]Phe binding. Values are means of duplicate determinations varying < 10%.
† ND, not determined.

polar lysine is added to the tripeptide f-Met-Leu-Phe. Phenylalanine in Position 4 increases binding activity 40-fold, whereas lysine, when linked to the tripeptide by either its alpha or epsilon nitrogen, results in a 10–30-fold decrease in activity. This decreased activity suggests that the lysine side chain, being quite basic, is repelled from a cationic site on the receptor. Phenylalanine, on the other hand, may occupy a hydrophobic pocket in the receptor similar to that proposed for phenylalanine when in Position 3.

Correlation of Binding Inhibition and Biologic Response

The ability of various N-formylated peptides to compete with f-Met-Leu-[^{3}H]Phe binding to rat neutrophils was compared with the ability of these same peptides, in the presence of cytochalasin B, to induce release of the lysosomal granule enzymes lysozyme and N-acetyl-β-D-glucosaminidase. As shown in Table 2, a greater than 20,000-fold range in enzyme-releasing activity is seen. The correlation coefficients, r, comparing the ability of the peptides to inhibit 50% of the specific f-Met-Leu-[^{3}H]Phe binding (ID_{50}) and the ability to cause half-maximal enzyme release (ED_{50}) for lysozyme and N-acetyl-β-D-glucosaminidase are $r = 0.83$ and $r = 0.74$, respectively. This strong correlation between the peptides' ability to compete for the receptor and to induce a biological response is also seen in their ability to cause superoxide production (Table 2) ($r = 0.95$). Boc-Phe-Leu-Phe-Leu-Phe, an antagonist of the chemotactic peptides,[3] gave no biologic response at a concentration of peptide almost two logs greater than required to displace 50% of specific f-Met-Leu-[^{3}H]Phe binding.

Discussion

The binding of the synthetic N-formylated oligopeptides to a specific receptor on the inflammatory cells' surface initiates a number of biochemical and physiologic processes, including chemotaxis, granule enzyme secretion, superoxide production, and initiation of the respiratory burst.[27] These various responses, when directed against invasive bacterial cells, allow the neutrophil and macrophage to perform their major function of defending the host against bacterial infection. Furthermore, the broad specificity of this receptor for the synthetic formylmethionyl peptides allows for a range of several orders of magnitude in biologic activity. This substantiates a physiologic role of this receptor in host defense.

Table 2—Comparison of Biologic Activity of Various Synthetic Oligopeptides

Peptide	Enzyme secretion (ED_{50})*		Superoxide (O_2^-) production (ED_{50})†
	Lysozyme	β-D-glucosaminidase	
f-Met-Leu-Phe	1.4×10^{-8}	2.2×10^{-8}	4.0×10^{-8}
Met-Leu-Phe	1.8×10^{-5}	5.6×10^{-5}	1.6×10^{-4}
2-Ethyl-hexanoyl-Leu-Phe	ND‡	2.4×10^{-6}	4.1×10^{-5}
f-Ala-Leu-Phe	ND	$>> 10^{-4}$	ND
f-Abu-Leu-Phe	ND	$>> 10^{-4}$	ND
f-Nle-Leu-Phe	ND	6.1×10^{-8}	9.9×10^{-8}
f-Met-Gly-Phe	3.0×10^{-5}	9.2×10^{-5}	3.9×10^{-5}
f-Met-Abu-Phe	4.0×10^{-7}	1.3×10^{-7}	2.6×10^{-7}
f-Met-Cys(Me)-Phe	4.3×10^{-6}	5.6×10^{-6}	ND
f-Met-Leu-Glu	ND	4.1×10^{-6}	3.4×10^{-6}
f-Met-Leu-Phe-Phe	4.6×10^{-9}	2.1×10^{-9}	ND
f-Met-Leu-Phe-α-Lys	1.0×10^{-7}	6.8×10^{-7}	2.3×10^{-7}
f-Met-Leu-Phe-ε-Lys	3.2×10^{-7}	6.6×10^{-7}	ND
Boc-Phe-Leu-Phe-Leu-Phe	$>> 10^{-4}$	$>> 10^{-4}$	$>> 7.7 \times 10^{-6}$

* ED_{50} is the concentration of peptide required to give 50% of the maximal f-Met-Leu-Phe-induced enzyme secretion, which ranged from 15% to 25% of the total enzyme present.
† ED_{50} is the concentration of peptide required to generate 50% of the maximal f-Met-Leu-Phe-induced superoxide production (maximal O_2^- production averaged 3.5 nmol per 2×10^6 cells).
‡ ND, not determined.

We have characterized the binding of f-Met-Leu-[^{3}H]Phe to the formyl peptide receptor on the rat neutrophil and several biologic responses, (enzyme secretion and superoxide production) that occur as a result of this peptide–receptor interaction. The rat neutrophils can be readily elicited to and recovered from the peritoneum in a highly enriched form and in abundant numbers. Furthermore, because the rat is used extensively in the investigation of the effects of a multitude of pharmacologic agents on inflammation and neutrophil function, both *in vivo*[28] and *in vitro*, we felt a detailed analysis of formyl peptide receptor on the rat neutrophil was warranted.

When binding studies were carried out at 24 C under conditions where dissociation of cell-bound peptides could occur (Figure 4), 65,000 receptor sites/cell could be calculated with a dissociation constant $K_D = 3.45 \pm 1.18 \times 10^{-8}$ M. Roch-Arveiller et al[13] have previously reported that rat neutrophils, recruited to the pleural cavity, possess 180,000 receptor sites/cell with a dissociation constant $K_D = 7 \times 10^{-8}$ M. The reason for this discrepancy in both the affinity and number of receptors is unclear; however, Roch-Arveiller et al[13] do not report the addition of sodium azide to their medium when performing binding studies on neutrophils with f-Met-Leu-[^{3}H]Phe for 30 minutes at 22 C. Under these conditions, some degree of receptor-mediated peptide endocytosis is likely to have occurred.[16,22]

In the binding studies we report, cells and f-Met-Leu-[^{3}H]Phe were incubated in medium containing both the metabolic inhibitor (2-deoxyglucose) and sodium azide, an agent known to block receptor endocytosis. Internalization of a fluorescent chemotactic peptide by human neutrophils is known to be energy-dependent and is blocked by agents that inhibit receptor endocytosis.[29] Under these same conditions both saturable and reversible binding of f-Met-Leu-[^{3}H]Phe could be demonstrated. However, a time-dependent loss of reversible binding is seen at 24 C, which occurs in the absence of additional peptide uptake, suggesting that a receptor-mediated event has occurred that restricts ligand dissociation. These results do not exclude the possibility that the irreversibly bound peptide was due to internalization of the receptor–ligand complex, which may still occur but at a much slower rate under the assay conditions described.

We have been unable to show an increase in the rate or extent of fluid-phase pinocytosis by rat PMNs, as measured by tritiated sucrose uptake, at 24 C after the addition of 1 μM f-Met-Leu-Phe. Under the conditions we describe, the uptake of ^{3}H-sucrose at 24 C was complete by 10 minutes (data not shown). This observation is in agreement with that of Fletcher et al,[30] who, using human neutrophils, found that nonspecific pinocytosis of extracellular fluid by the cells could not explain either the increased uptake or nondisplaceability of f-Met-Leu-[^{3}H]Phe. These observations are in contrast to those reported by Sullivan and Zigmond,[16] who observed, for rabbit PMNs, a fourfold increase in the rate of pinocytosis at both 4 C and 37 C following the addition of a similar concentration of chemotactic peptide. Whether the difference in species may explain these discrepant results is unknown; however, with the rat neutrophil, the irreversibility of formyl peptide binding at 24 C would not appear to be due to receptor-mediated fluid phase pinocytosis.

Several biologic responses occur in the neutrophil as a result of binding to the formyl peptide receptor.[27] In addition, the biologic activity of these peptides is proportional to their binding. Our studies extend the multifunctional nature of this receptor on the rat neutrophil. We have observed this same high correlation between binding and biologic activity (enzyme secretion, superoxide production) with the rat neutrophil, as reported in several other species.[2,9–12,20] The ED_{50}s for enzyme secretion and superoxide production are nearly equal to the ID_{50} for binding, suggesting that a large percentage of these receptors must be occupied for the subsequent biologic responses to be triggered. This observation has also been seen in other species.[9,31]

With regard to the specificity of the rat neutrophil formyl peptide receptor, strong similarities exist across species lines. Most obvious is the absolute requirement of the NH_2-terminal formyl group. In addition, methionine in Position 1 and phenylalanine in Position 3 confirm maximal binding for the tripeptide. The aliphatic straight chain α-aminobutyric acid derivative has approximately the same activity a does the gamma-branched leucine when in Position 2. The most striking finding of these studies, however, was the 40-fold increase in binding and biologic activity that resulted when phenylalanine was added in Position 4. This same effect is seen with the rabbit neutrophil.[4,5] This dramatic increase in binding activity is possibly due to a greater portion of the binding site being occupied.[4] Alternatively, increased stabilization of the optimal conformation of the peptide that fits the receptor or in the proportion of molecules that fit the receptor may result from the additional intramolecular hydrogen bonding with phenylalanine in Position 4.

We conclude from these studies that the fine specificity of the formyl peptide receptor has been conserved across species lines. The rat neutrophil has

65,000 receptor sites/cell for f-Met-Leu-[^{3}H]Phe with a dissociation constant (K_D) of 3.4×10^{-8} M. Our ability to recover large numbers of highly purified neutrophils from the rat will greatly facilitate further in-depth studies of the formyl peptide receptor after pharmacologic manipulation both *in vivo*[28] and *in vitro*.

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