The significance of functional receptor heterogeneity in the biological responses of the rabbit neutrophil to stimulation by chemotactic formyl peptides

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The characteristics of binding to the chemotactic receptors on rabbit peritoneal neutrophils were examined for seven formyl peptide analogues. These receptor-binding characteristics were compared with the abilities of the analogues to induce the biological responses of degranulation and chemotaxis. Five of the analogues showed distinct functional heterogeneity in their receptor-binding patterns, whereas the two most potent compounds displayed homogeneous binding patterns. The relative potencies of the formyl peptide analogues for stimulation of degranulation correlated well with their relative potencies for high-affinity, but not low-affinity, binding. The biphasic patterns for stimulation of chemotactic migration were similar for the less potent analogues, and their potencies paralleled those for both degranulation and receptor binding. In contrast, the most potent analogues induced a greater maximal extent of chemotactic migration than the other compounds, but displayed a lower than expected potency (i.e. they required higher than expected concentrations). These anomalies in the patterns of the chemotactic response cannot be reconciled with a simple receptor model comprising two independent classes of receptors. Instead, a model comprising interconvertible states of different affinities is proposed. The state of higher affinity appears to play a central role in initiation of both degranulation and chemotaxis. The more potent formyl peptide analogues are thought to stabilize an activated, higher-affinity, state of the receptor; this can explain their greater efficacy in stimulating chemotaxis. The proposed model may also be applicable to other receptors that are coupled by a guanine-nucleotide-binding regulatory protein to their associated effector.

INTRODUCTION

The neutrophil plays a major role in the body's defence mechanism against infectious micro-organisms. It responds by moving towards, engulfing and digesting such foreign pathogens. Binding of a chemotactic factor to a specific receptor on the neutrophil surface triggers the diverse repertoire of responses of this cell [1–4]. Although much progress has been made towards characterizing the receptors for several chemotactic substances, particularly the formyl peptides, little is as yet known about the differential control of the various cellular responses.

Many studies of the chemotactic formyl peptide receptors on the neutrophil have provided evidence of divergence from the simple binding pattern predicted by the Law of Mass Action for binding to a single class of similar independent receptors of fixed affinity [5–14]. The receptor-binding pattern is thus regarded as functionally heterogeneous. Different types of studies, however, have yielded evidence of different forms of heterogeneity [5,7,9,10,13]. There is thus no consensus about the most appropriate receptor model to explain the observed binding pattern. Nor is it clear what role, if any, the functional receptor heterogeneity plays in determining the diverse biological responses of the neutrophil upon stimulation by a chemotactic factor.

The principal explanation for binding heterogeneity such as that observed is the existence of high- and low-affinity forms of

the receptor. These may be interconvertible states of a single receptor [7,15–17], or distinct and totally independent receptor classes [5,18]. One proposal for the neutrophil is that the high-affinity form of the receptor may be responsible for activation of some biological functions, notably chemotaxis, with the low-affinity form responsible for other functions, e.g. degranulation [1,19–21]. Similar proposals have been made to explain the differential activation of a range of biological responses in several other cell types and with several other receptor agonists. The only evidence to date to support this hypothesis for the neutrophil, however, is derived from studies of the influence of various perturbations of the cell on both the receptor-binding pattern and the biological responses for a single chemotactic formyl peptide, the prototypical compound *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe).

The study described in this paper represents a different approach to test this hypothesis. Instead of manipulating the properties of the cell, the responses of intact neutrophils were examined for a series of formyl peptide analogues. The prototypical compound and six analogues of widely differing potency were tested and compared in detail for their receptor-binding patterns and abilities to stimulate degranulation and chemotaxis. The experimental data were evaluated on the basis of an independent 'two-binding-site' model for the receptors, primarily because this model is currently the only one amenable to a thorough and complete mathematical analysis.

Abbreviations used: ED₅₀, effective dose at half-maximal response; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; fMet-Leu-Phe-NHBzl, fMet-Leu-Phe benzylamide; fMet-Leu-Phe-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanyl-L-phenylalanine; fNle-Leu-Phe, *N*-formyl-L-norleucyl-L-leucyl-L-phenylalanine; fNle-Leu-Phe-Tyr, *N*-formyl-L-norleucyl-L-leucyl-L-phenylalanyl-L-tyrosine; fNva-Leu-Phe, *N*-formyl-L-norvalyl-L-leucyl-L-phenylalanine; fVal-Leu-Phe, *N*-formyl-L-valyl-L-leucyl-L-phenylalanine; G-protein, guanine-nucleotide-binding regulatory protein; *K*_d, equilibrium dissociation constant.

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MATERIALS AND METHODS

Chemicals

Cytochalasin B was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); shellfish glycogen, crystalline BSA, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide and the chemotactic formyl tripeptides fMet-Leu-Phe and *N*-formyl-L-norleucyl-L-leucyl-L-phenylalanine (fNle-Leu-Phe) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); *N*-formyl-L-norleucyl-L-leucyl-L-phenylalanyl-L-tyrosine (fNle-Leu-Phe-Tyr) was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). The four remaining chemotactic formyl peptides were synthesized by a rapid mixed anhydride method and purified by crystallization, as described previously [22,23].

Each of the non-radioactive formyl peptides showed a purity in excess of 90% when analysed by t.l.c. in butan-1-ol/acetic acid/water (4:1:1, by vol.) containing 0.2% (v/v) 2-mercaptoethanol [12,24]. The purity of each of the four synthesized formyl peptides was confirmed on two other t.l.c. systems and by quantitative amino acid analysis [22].

Radiochemicals

The radiolabelled formyl peptides fMet-Leu-[^3H]Phe (48–60 Ci/mmol), [^{35}S]Met-Leu-Phe (56–780 Ci/mmol) and fNle-Leu-[^3H]Phe (41–47 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.); fNle-Leu-[^3H]Phe-Tyr (66 Ci/mmol) was a gift from New England Nuclear; and [^3H]N-formyl-L-methionyl-L-leucyl-L-phenylalanyl-L-phenylalanine (fMet-Leu-Phe-Phe), of corrected specific radioactivity 21 Ci/mmol [25], was obtained from Amersham Corp.

Before use for receptor studies, fMet-Leu-[^3H]Phe, [^{35}S]Met-Leu-Phe and [^3H]fMet-Leu-Phe-Phe were repurified by t.l.c. in butan-1-ol/acetic acid/water (4:1:1, by vol.) containing 0.2% 2-mercaptoethanol, using the procedure described previously [24]. Repurification was repeated at 3-month intervals. No repurification was necessary, however, with the purer and more stable norleucyl analogues [12].

The quality of each radioligand was established using the methods described previously for radiolabelled fMet-Leu-Phe, including radiochemical analysis by t.l.c., assessment of receptor reactivity and bioassay [24]. The results of these quality assessments have already been published [12,24,25], with the exception of the bioassay data for fNle-Leu-[^3H]Phe-Tyr. The biological potency of the latter radioligand was $113 \pm 15\%$ (mean $\pm$ S.E.M.; from four studies comparing the degranulation response induced by the radioligand with that induced by non-radioactive fNle-Leu-Phe-Tyr). All five radioligands were thus essentially pure and equivalent to their non-radioactive counterparts; all were suitable for detailed receptor studies [24,26].

Rabbit neutrophils

Rabbit peritoneal neutrophils were collected 5–7 h after the injection of 300 ml of sterile shellfish glycogen (1 mg/ml) in 150 mM-NaCl, as described previously [12,27]. Neutrophils elicited in this manner have previously proved suitable for detailed characterization of the biological responses to and receptor binding of chemotactic formyl peptide analogues [12,22,23]. The neutrophils were washed twice in Mg^{2+} -free Hanks' balanced salt solution containing 1.7 mM- CaCl_2 , D-glucose (1 mg/ml) and 10 mM-Hepes, pH 7.2. They were resuspended in the same solution containing BSA (0.1 mg/ml) for the receptor-binding and degranulation studies, and in the same solution containing 0.7 mM- MgSO_4 and BSA (0.5 mg/ml) for the chemotaxis studies.

Receptor-binding assay

Binding of the various formyl peptides to the chemotactic receptors on intact rabbit neutrophils was assessed by the silicone oil assay described previously [12,25]. In brief, the usual procedure involved the layering of 50 μl of formyl peptide solution (radioligand on its own or together with a non-radioactive ligand) at 4 $^\circ\text{C}$ over 500 μl of Versilube F50 silicone oil (General Electric, Silicone Products Division, Waterford, NY, U.S.A.) in a microcentrifuge tube; the binding reaction was started by the careful addition of 50 μl of neutrophil suspension (2×10^7 cells/ml, final concentration). Different reaction volumes (200 μl in total) and neutrophil concentrations (5×10^8 cells/ml), however, were used in the studies with [^3H]fMet-Leu-Phe-Phe. The reaction was stopped after 15 min at 4 $^\circ\text{C}$ by centrifugation (13000 g, 2 min), and the radioactive content of the cell pellet was determined by scintillation counting [12]. All binding measurements were performed in duplicate, and each binding curve comprised 14 or 15 dose points spanning an approximate 1000-fold range.

The receptor-binding studies undertaken were of two types: direct studies of the saturability of binding at equilibrium with increasing concentrations of a tritiated radioligand [12], and indirect studies in which the binding of a non-radioactive analogue was deduced from its ability to competitively inhibit the binding of a tracer concentration (5×10^{-11} M) of [^{35}S]Met-Leu-Phe [25]. Each study comprised a comparison of the binding characteristics of two or three of the formyl peptide analogues; fMet-Leu-Phe was always included as one of the competing ligands whenever the competitive binding approach was adopted. For three of the formyl peptides (fMet-Leu-Phe, fNle-Leu-Phe and fMet-Leu-Phe-Phe), studies of both types were performed and the resulting estimates of binding affinity were indistinguishable (for fMet-Leu-Phe, six direct binding studies yielded geometric mean affinity values, based on an independent two-binding-site model, of 4.9×10^{-10} and 3.7×10^{-9} M for the high- and low-affinity sites respectively, whereas 18 competitive binding studies yielded values of 5.0×10^{-10} and 4.5×10^{-9} M respectively; $P > 0.5$ and $P > 0.2$, t tests after logarithmic data transformation). Only direct binding studies were performed with fNle-Leu-Phe-Tyr, and only competitive binding studies with the remaining three analogues. That the incubation time (15 min) used in these studies was sufficient to allow equilibrium binding has been shown previously for both the most potent and the least potent of the radioligands [25,28]; the lack of any change in competitive inhibition pattern for *N*-formyl-L-norvalyl-L-leucyl-L-phenylalanine (fNva-Leu-Phe) when the incubation time was increased to 1 h further implied that 15 min was sufficient for equilibration even with this formyl peptide analogue (one of the least potent).

The data for all studies were analysed using the PREBIND computer program in conjunction with a version of the LIGAND program [29,30] that had been adapted for use on the IBM-PC range of computers by Dr. G. McPherson [31,32]. [Copies of the PREBIND program, written in QuickBASIC for the IBM-PS/2 by one of the authors (J.C.K.), are available on request.] The binding data were analysed on the basis of an independent two-binding-site model with receptors of fixed affinity. The general analytical approach has been described in detail in previous reports. Its most important features are the correction of all binding data for the receptor reactivity of the radioligand, the weighting of data points during curve fitting on the basis of a constant percentage error, the simultaneous and constrained fitting of binding curves for all ligands (two or three) included within a particular study, and the treatment of non-specific binding as a computer-fitted parameter [12,25,26]. It should be emphasized that the choice of an independent two-binding-site

model for data analysis is an arbitrary one, governed more by the availability of a suitable computer program than by the appropriateness of this model to the biological system. Equilibrium-binding curves calculated on the basis of this model, however, are essentially indistinguishable from those calculated on the basis of a model comprising two interconvertible states differing in affinity [15,16], so the quality of the fit to one particular model should not be regarded as evidence to exclude the other. On the contrary, a good fit to one model also implies a good fit to the other.

Degranulation assay

The stimulation by chemotactic formyl peptide of the release of *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30) from the granules of rabbit neutrophils was assessed by the procedure described previously [24,25]. In brief, rabbit neutrophils (5×10^6 cells/ml) were incubated for 5 min at 37 °C with a series of concentrations of formyl peptide in the presence of cytochalasin B ($5 \mu\text{g/ml}$). The β -glucosaminidase released from the cells was assayed using the chromogenic substrate *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide. All measurements were performed in duplicate, and each dose-response curve comprised 9 or 10 dose points spanning an approximate 1000-fold range. The dose-response curves were analysed using the ENZREL computer program [24,25].

Chemotaxis assay

Formyl peptide stimulation of chemotaxis by rabbit neutrophils was assessed by a modification of the Boyden chamber assay [33]. Each lower well of the multi-chamber blind-well chemotaxis plate was filled with 150 μl of formyl peptide solution (a series of concentrations), and 150 μl of neutrophil suspension (10^6 cells/ml) was placed above the filter (8 μm pore size and approx. 100 μm thickness; Millipore Corp., Bedford, MA, U.S.A.). The chemotaxis chambers were incubated for 1 h at 37 °C. The cells were then fixed *in situ* by the addition of 250 μl of ethanol to each well, this procedure ensuring that non-migrating cells remained attached to the filter. Cell counts were performed on haematoxylin-stained filters at the cell monolayer atop the filter and at 10 μm depth increments, using an Olympus (Lake Success, NY, U.S.A.) model CH microscope (200 $\times$ overall magnification), an Ikegami (Maywood, NJ, U.S.A.) video camera, a Hughes Aircraft Corp. (Los Angeles, CA, U.S.A.) model 794 image enhancer and an Optomax (Burlington, MA, U.S.A.) model 40.10 image analyser. Each dose-response curve comprised 8 or 9 dose points spanning an approximate 1000-fold range. Five geometrically selected, non-overlapping, fields were counted on each of the two filters at each dose. Cell counts were analysed using the CHEMOTAXIS and CURVMAKE computer programs. [Copies of these programs, written by one of the authors (J. C. K.), in Applesoft Basic for the Apple II+ computer and in QuickBASIC for the IBM-PS/2 respectively, are available on request.] The chemotaxis data are presented as average migration distances (cells that remained at the monolayer being included in the total cell number used to calculate this average).

RESULTS

Receptor-binding characteristics

Direct binding studies with fMet-Leu-[^3H]Phe, fNle-Leu-[^3H]Phe and fNle-Leu-[^3H]Phe-Tyr revealed functional heterogeneity in the patterns of binding of these formyl peptides to the receptors on intact rabbit neutrophils, as previously reported [12], i.e. the binding patterns diverged from the predictions of the Law of Mass Action for binding to a single class of similar, independent receptors of fixed affinity. The binding data were,

instead, consistent with the presence of two independent classes of receptors differing in affinity (Table 1). Consistency with this model, however, implies that the data should also be consistent with a model comprising two interconvertible states differing in affinity; these alternative models should not be regarded as mutually exclusive (see the Materials and methods and Discussion sections).

Competitive binding studies were performed with two formyl peptide analogues of lower biological potency, fNva-Leu-Phe and *N*-formyl-L-valyl-L-leucyl-L-phenylalanine (fVal-Leu-Phe). Each of these analogues was able to compete with [^3S]Met-Leu-Phe for binding to both of the postulated classes of receptors (Fig. 1a). The competitive binding patterns were consistent with an independent two-binding-site model [$P > 0.5$ for fNva-Leu-Phe and $P > 0.1$ for fVal-Leu-Phe; partial *F* test (see [25] for method of calculation)], and the calculated binding affinities for each analogue differed between the two binding sites (Table 1).

In contrast, we have previously reported an essentially homogeneous binding pattern (consistent with a one-binding-site model) for a highly potent formyl peptide analogue, fMet-Leu-Phe-Phe [25]. This conclusion was derived from both direct and competitive binding studies. A similar pattern was found in the present study for competitive binding by another very potent analogue, fMet-Leu-Phe benzylamide (fMet-Leu-Phe-NHBzl). Like fMet-Leu-Phe-Phe, this compound competed with almost

Table 1. Equilibrium dissociation constants for binding of chemotactic formyl peptide analogues to the receptors on rabbit neutrophils

Equilibrium binding data were analysed by the PREBIND and LIGAND computer programs on the basis of an independent two-binding-site model with receptors of fixed affinity. Composite analysis was performed on the data for all formyl peptide analogues (two or three) included within a given experiment, using the analytical methods described previously [12,25]; all formyl peptides within an experiment were thereby constrained to bind to the same sites (i.e. to have the same proportions of high-affinity and low-affinity sites). Dissociation constants are geometric mean values from all receptor-binding experiments with each analogue (the range of values being indicated in Figs. 3 and 5). The data encompass both direct (saturation) and indirect (competitive) binding studies. All competitive binding studies used [^3S]Met-Leu-Phe (5×10^{-11} M) as the tracer ligand; each such study invariably included a competition curve with non-radioactive fMet-Leu-Phe (as well as competition curves with other chosen non-radioactive analogues), and affinity values for the radioligand were based on this self-competition curve during the constrained curve-fitting process (assuming equivalence between ^3S -labelled and non-radioactive fMet-Leu-Phe [24]). The high-affinity sites are the less numerous binding sites that show a higher binding affinity for most formyl peptide analogues. fMet-Leu-Phe-NHBzl is anomalous in that the less numerous sites showed, on average, a lower binding affinity than the more numerous sites. Each neutrophil was estimated to have 16000 high-affinity and 78000 low-affinity sites (geometric mean values). The affinity ratio is the ratio of the dissociation constant for the low-affinity sites to that for the high-affinity sites. *Affinity ratio not significantly different from unity ($P > 0.1$, partial *F* test).

Formyl peptide	<i>n</i>	Dissociation constant (M)		
		High-affinity sites	Low-affinity sites	Affinity ratio
fMet-Leu-Phe-Phe	6	7.9×10^{-11}	1.6×10^{-10}	2.0
fMet-Leu-Phe-NHBzl	6	9.3×10^{-11}	5.9×10^{-11}	0.6*
fNle-Leu-Phe-Tyr	6	2.1×10^{-10}	2.0×10^{-9}	9.6
fMet-Leu-Phe	24	5.0×10^{-10}	4.3×10^{-9}	8.5
fNle-Leu-Phe	10	1.4×10^{-9}	1.7×10^{-8}	12.6
fNva-Leu-Phe	4	6.0×10^{-8}	3.7×10^{-7}	6.2
fVal-Leu-Phe	4	1.0×10^{-7}	5.7×10^{-7}	5.4

Fig. 1. Characteristics of binding of chemotactic formyl peptide analogues to the receptors on rabbit neutrophils

Competitive inhibition of [35 S]Met-Leu-Phe binding to neutrophils (a) by non-radioactive fMet-Leu-Phe (O) and fVal-Leu-Phe (▼), and (b) by non-radioactive fMet-Leu-Phe (O) and fMet-Leu-Phe-NHBzl (▲). The results are from typical studies. Each point is the mean of duplicate determinations whose coefficient of variation averaged 5.0% and 6.7% respectively. The lines represent the optimal fits to an independent two-binding-site model with receptors of fixed affinity, subject to the constraint that the two formyl peptides in each study interact with the same binding sites. The PREBIND and LIGAND computer programs were used to analyse the binding data, and the lines corresponding to the fitted parameters were constructed by the CURVMAKE program. The proportion of high-affinity receptors was estimated to be 20% and 44% in the studies shown in (a) and (b) respectively. The two binding sites differed significantly in affinity for fMet-Leu-Phe (affinity ratios of 6.2 and 9.9 respectively; $P < 0.005$ in each case, partial F test) and fVal-Leu-Phe (affinity ratio of 5.6; $P < 0.005$), but not for fMet-Leu-Phe-NHBzl (affinity ratio of 1.6; $P > 0.1$).

equal affinity for binding of [35 S]Met-Leu-Phe to both classes of binding sites (Table 1). The different binding pattern for this analogue compared with the less potent compounds was readily apparent from its much steeper competition curve (Fig. 1b).

The equilibrium dissociation constant (K_d) for high-affinity binding had a range of 1300-fold between the least potent (fVal-Leu-Phe) and the most potent (fMet-Leu-Phe-Phe) of the seven formyl peptide analogues, whereas the K_d for low-affinity binding varied 9700-fold (Table 1). The total binding capacity of the neutrophil, for both classes of sites in an independent two-

Fig. 2. Stimulation of neutrophil degranulation by chemotactic formyl peptide analogues

The results are from a typical study of the release of β -glucosaminidase upon stimulation by fNle-Leu-Phe (■), fMet-Leu-Phe (O) and fMet-Leu-Phe-Phe (●). Each point is the mean of duplicate determinations whose coefficient of variation averaged 4.3%. The lines represent the optimal log-logistic curve fits, constrained to be parallel and to have equal maxima and minima for all three formyl peptides. These data were analysed by the ENZREL computer program, and the lines corresponding to the fitted parameters were constructed by the CURVMAKE program.

binding-site model, did not differ between the analogues ($P > 0.1$, partial F test); there were estimated to be 94000 (geometric mean) formyl peptide receptors on each neutrophil, and 17% (geometric mean) of these were of the high-affinity class.

Degranulation characteristics

Dose-response curves for stimulation of degranulation by each formyl peptide analogue were assessed in association with the receptor-binding studies (using the same neutrophil preparations). All seven analogues induced the same maximal extent of β -glucosaminidase release, and the dose-response curves were parallel (Fig. 2). The geometric mean slope of the computer-fitted log-logistic dose-response curves was 1.50; this is equivalent to a difference of 19-fold between doses causing responses of 10% and 90% of maximal release (compared with an 81-fold difference for receptor binding with a non-cooperative one-binding-site model). The effective dose at half-maximal response (ED_{50}) for degranulation showed a 950-fold difference between

Table 2. Characteristics of the degranulation and chemotactic responses of rabbit neutrophils to formyl peptide analogues

Dose-response curves for β -glucosaminidase release were analysed by the ENZREL computer program. Composite analysis was performed on the data for all formyl peptide analogues included within a given experiment. Chemotactic dose-response curves were analysed as symmetrical, biphasic, log-logistic curves by a computer-assisted least-squares approach using the CURVMAKE program. ED_{50} values are geometric mean values from the various experiments (the range of values being indicated in Fig. 3 or Fig. 5). The maximal migration is the average migration distance (mean $\pm$ S.E.M.) at the peak of the biphasic chemotactic dose-response curve. For comparison, the migration in the absence of formyl peptide averaged $1.9 \pm 0.5 \mu\text{m}$.

Formyl peptide	Degranulation		Chemotactic response		
	<i>n</i>	ED_{50} (M)	<i>n</i>	ED_{50} (M)	Maximal migration (μm)
fMet-Leu-Phe-Phe	6	4.0×10^{-11}	4	1.1×10^{-10}	31.3 ± 4.0
fMet-Leu-Phe-NHBzl	6	5.5×10^{-11}	4	1.0×10^{-10}	26.9 ± 4.3
fNle-Leu-Phe-Tyr	6	1.5×10^{-10}	4	1.1×10^{-10}	18.3 ± 2.5
fMet-Leu-Phe	24	3.2×10^{-10}	7	1.3×10^{-10}	12.7 ± 3.3
fNle-Leu-Phe	10	1.2×10^{-9}	4	4.5×10^{-10}	15.4 ± 3.0
fNva-Leu-Phe	4	1.5×10^{-8}	4	9.6×10^{-9}	15.7 ± 1.5
fVal-Leu-Phe	5	3.8×10^{-8}	4	1.1×10^{-8}	12.5 ± 2.1

Fig. 3. Potency comparison between receptor binding and degranulation

Correlation between the relative potencies of the formyl peptide analogues for inducing degranulation and their potencies for binding to the sites of (a) high affinity and (b) low affinity. All binding K_d values for this comparison were based on an independent two-binding-site model with receptors of fixed affinity. The data points are geometric mean values from the various experiments (the number of experiments on which each mean is based is shown in Tables 1 and 2); the error bars show approx. 67% confidence intervals for these means (equivalent to mean $\pm$ S.E.M. with logarithmic transformation). The lines shown are those of best fit to the equation $\log y = \log x + \log a$ (45° line of best fit with logarithmic scaling), with each data point weighted according to the number of experiments performed; all formyl peptides were included in fitting the line to the high-affinity K_d estimates, but fMet-Leu-Phe-NHBzl and fMet-Leu-Phe-Phe were excluded for the low-affinity fit. (It should be noted that the more general regression equation $\log y = b \log x + \log a$ is inappropriate for this type of potency comparison of logarithmically transformed data.)

the least potent (fVal-Leu-Phe) and the most potent (fMet-Leu-Phe-Phe) analogues (Table 2).

Correlation of degranulation with receptor binding

The ED_{50} values for the degranulation response for the seven analogues were correlated closely with their K_d values for high-affinity binding (Fig. 3a): the experimental data (logarithmically transformed) did not deviate significantly from a 45° line of best fit ($P > 0.5$, analysis of covariance). In absolute terms, however, the ED_{50} for degranulation was consistently slightly lower, by a factor averaging 1.7, than the high-affinity K_d estimate.

For five of the analogues a similar correlation was found between the ED_{50} for degranulation and the K_d for low-affinity binding, although in this case there was a 14-fold difference in absolute potencies (Fig. 3b). This correlation, however, did not extend to the two most potent analogues, fMet-Leu-Phe-Phe and fMet-Leu-Phe-NHBzl. The experimental data points for each of the latter analogues deviated substantially from the 45° line of fit through the remaining five data points ($P < 0.001$ in each case; analysis of covariance).

Characteristics of the chemotactic response

The dose-response patterns for stimulation of chemotaxis were also assessed for each of the seven analogues. The ED_{50} values for this response varied only 110-fold between the least potent (fVal-Leu-Phe) and the most potent (fMet-Leu-Phe-NHBzl) analogue. The four weakest compounds in terms of degranulation potency (fMet-Leu-Phe, fNle-Leu-Phe, fNva-Leu-Phe and fVal-Leu-Phe) induced the same maximal extent of migration ($P > 0.5$, analysis of variance; Table 2); their ED_{50} values for the chemotactic response approximately paralleled those for stimulation of degranulation. In contrast, significantly greater extents of maximal migration (greater efficacies) were found with both fMet-Leu-Phe-Phe and fMet-Leu-Phe-NHBzl ($P < 0.001$ and $P < 0.005$ respectively; Fig. 4 and Table 2). In terms of ED_{50} , the latter analogues were only marginally, if at all, more potent than fMet-Leu-Phe in stimulation of chemotaxis.

Fig. 4. Stimulation of neutrophil chemotaxis by formyl peptide analogues

The results are from a typical study of chemotaxis induced by fNva-Leu-Phe ($\square$), fMet-Leu-Phe ($\circ$) and fMet-Leu-Phe-Phe ($\bullet$). Each point is the mean $\pm$ S.E.M. of ten determinations (five fields on each of two filters). The CHEMOTAXIS and CURVMAKE computer programs were used to analyse these data and construct the symmetrical, biphasic, log-logistic dose-response curves that yielded the optimal fits.

The behaviour of fNle-Leu-Phe-Tyr was intermediate between the two more potent and four less potent analogues (Table 2), although in this case the increase in efficacy (compared with the weakest analogues) was not statistically significant ($P > 0.2$).

The increase in maximal migration seen with the more potent analogues primarily reflected a greater degree of migration (or improved directionality) by each cell; the proportion of cells migrating was only slightly elevated (averaging 75% at the peak of the dose-response curve for fMet-Leu-Phe-Phe compared with 55% for fVal-Leu-Phe).

Correlation of chemotaxis with receptor binding

The ED_{50} estimates for stimulation of chemotaxis by the seven analogues did not correlate well with their K_d values for either

Fig. 5. Potency comparison between receptor binding and chemotaxis

Correlation between the relative potencies of the formyl peptide analogues for inducing chemotaxis and their potencies for binding to the sites of (a) high affinity and (b) low affinity. Data were analysed and are displayed in the same way as in Fig. 3. The 45° lines of best fit (for the equation $\log y = \log x + \log a$) were constructed using only the four analogues of lowest potency (fVal-Leu-Phe, fNva-Leu-Phe, fNle-Leu-Phe and fMet-Leu-Phe), i.e. those that induced the same maximal extent of chemotactic migration.

high- or low-affinity binding (Fig. 5). This is a somewhat unsatisfactory comparison, however, in view of the different chemotactic efficacies of some analogues. If the comparison is restricted to the four analogues of similar efficacy (those of lowest potency), the ED_{50} estimates correlated well with both high- and low-affinity K_d values: on average, the ED_{50} for stimulation of chemotaxis was lower by a factor of about 4.6 than the K_d for high-affinity binding, and it was about 38-fold lower than the K_d for low-affinity binding. In contrast, for the two most potent analogues, fMet-Leu-Phe-Phe and fMet-Leu-Phe-NHBzl, the ED_{50} for the chemotactic response approximately equalled the K_d for high-affinity binding.

DISCUSSION

The present observations confirmed the functional heterogeneity of the binding of chemotactic formyl peptides to their receptors on the rabbit neutrophil [12], and extended this pattern to analogues of much lower potency than the prototypical compound fMet-Leu-Phe. This pattern of heterogeneous binding, however, did not extend to the two most potent analogues (fMet-Leu-Phe-Phe and fMet-Leu-Phe-NHBzl). The finding of functional heterogeneity suggests the existence of high- and low-affinity forms of receptor; different models postulate that these forms are either distinct, totally independent receptor classes of fixed affinity [18], or interconvertible states of a single receptor [15,16]. Two models of interconvertibility have been proposed: the ternary complex [16] and negative co-operativity [15] models. The present analysis utilized a model of independent receptors of fixed affinity [18], although the sole basis for choosing this model was its amenability to a thorough and complete mathematical evaluation. The binding data for all seven formyl peptides were consistent with this model; typically, 17% of the total cell complement of receptors were of the high-affinity class and the remaining 83% of low affinity; these two receptor classes generally differed about 10-fold in their affinity for most chemotactic formyl peptides, but did not differ in affinity for the two most potent analogues (Table 1).

Comparison of the binding and degranulation data revealed a good correlation between the K_d values for the high-affinity site and the ED_{50} estimates for the latter response (Fig. 3a). A much

worse correlation was found with the low-affinity K_d values (Fig. 3b). The logical interpretation of these data is thus that the high-affinity sites are the receptors that initiate degranulation. The slope of the degranulation dose-response curves (log-logistic slope factor of 1.50) also implies the presence of only a limited number of 'spare receptors'; this is consistent with a primary role for the high-affinity sites in initiating degranulation. (The 1.7-fold receptor excess implied by the ratio of the high-affinity K_d value to the biological ED_{50} should correspond to a slope factor of 1.52, whereas a slope factor of 1.95 would be expected if the low-affinity sites govern degranulation.) The present results thus contrast with those of earlier studies involving pharmacological manipulations of neutrophil behaviour [19–21]. Those earlier studies suggested that the low-affinity receptors (or the low-affinity state) might initiate some neutrophil functions, including degranulation. Those studies are difficult to interpret, however, because the treatments to which the neutrophils were subjected (ethanol, antibiotics, dithiothreitol) are by no means specific; they could, through an effect on plasma membrane fluidity, influence the biochemical pathways at several steps beyond the receptor [34–37], as well as at this step.

This comparison between receptor-binding and biological data may be influenced by two factors that are intrinsic to all such comparisons: the need to perform the assays at different temperatures, and the difference in the temporal nature of these processes. Data from other studies, however, imply that neither factor should invalidate the comparison. The present binding studies were performed at 4 °C because receptor processing complicates interpretation of studies on intact cells at 37 °C [28,38,39]. Studies on isolated plasma membranes, however, have shown the binding pattern and K_d estimates for fMet-Leu-Phe to be little affected by temperature [40], and to be similar to those found with intact neutrophils at 4 °C [5,10]. Furthermore, Sklar *et al.* have shown the one-way dissociation rate constants to be similar for formyl peptide agonists of widely differing potencies [41]; differences in the equilibrium K_d values thus closely reflect differences in the one-way association rate constants that govern the initial binding rate.

The four weakest compounds studied (fMet-Leu-Phe, fNle-Leu-Phe, fNva-Leu-Phe and fVal-Leu-Phe) induced a chemotactic response at lower doses than those at which they induced

Fig. 6. Receptor models to explain the patterns of formyl peptide binding and stimulation of both degranulation and chemotaxis

(a) A conceptual model of interconvertible affinity states. Biochemical signals for the stimulation of degranulation (left) and chemotaxis (right) are shown for two different extents of receptor occupancy (nominally, 10% and 50% occupancy) by either a less potent analogue (e.g. fMet-Leu-Phe; broken lines) or a very potent analogue (e.g. fMet-Leu-Phe-Phe; continuous lines). The vertical axis represents signal magnitude and the horizontal axis represents time. The signals are shown at two different stages in the appropriate biochemical pathways, i.e. before and after the limiting step in the amplification process. The first of these signals reflects the relevant signal from the effector; the other is a measure of the final biological response. [Although the effector signals are presumably the same for the two responses, only the initial portion of this signal is considered as the relevant part for the rapid degranulation response (see the Discussion section).] The signal for degranulation is shown as non-saturable (i.e. there are few spare receptors); that for chemotaxis is saturable in the case of the brief signal from a less potent analogue, but not in the case of the prolonged signal from a very potent analogue. Illustrated in the Figure is one way in which this differential saturability might arise, i.e. by way of a limit on the rate of increase of the amplified signal. (b) A biochemical counterpart to the conceptual model. The receptor (R) and its associated G-protein (subunits G_α and $G_{\beta\gamma}$) can exist in four different states. The receptor on its own (states I and IV) has a low binding affinity for the formyl peptides; its affinity is high when associated with either the complete G-protein (II) or the $G_{\beta\gamma}$ subunit alone (III). The resting state is considered to comprise an equilibrium between states I and II, with the balance probably favouring state I. States III and IV are both activated states in which the free G_α subunit is able to couple with the effector. (The mechanism shown is a simplification and, for clarity, omits the reactions between G_α and the guanine nucleotides GDP and GTP.)

a degranulation response, as previously reported for many formyl peptide agonists [1,22,42]. In contrast, the two most potent compounds (fMet-Leu-Phe-Phe and fMet-Leu-Phe-NHBzl) showed slightly higher ED_{50} values for stimulation of chemotaxis than of degranulation. These two most potent analogues thus displayed three anomalies compared with the less potent analogues: a homogeneous receptor-binding pattern (Fig. 1b and

Table 1), a greater maximal extent of stimulated migration (greater efficacy; Fig. 4) and a lower chemotactic potency (higher ED_{50}) than expected from the degranulation potency (Table 2). Two other groups have also shown increased chemotactic efficacy for very potent chemotactic formyl tetrapeptides [43,44]. The third most potent analogue in the present study (fNle-Leu-Phe-Tyr) also tended to show the same anomalies, albeit to a much lesser degree. These anomalies for the potent analogues cannot be reconciled with an independent two-binding-site model with receptors of fixed affinity; an alternative model of interconvertible affinity states thus appears more appropriate. It is probable, moreover, that the anomalies in chemotactic response are related to the anomaly in binding. The lack of any corresponding anomalies in the degranulation response is presumably related to the different nature of this response, most likely to its rapidity [45,46] compared with the prolonged time course of the chemotactic response [47,48]. Although the latter response requires receptor recycling [49,50], it is unlikely, in view of the differences in binding patterns in the absence of recycling (at 4 °C), that the anomalies in this response are due solely to anomalous recycling of receptors occupied by the potent analogues.

Although the present binding data did not show any significant deviations from an independent two-binding-site model with receptors of fixed affinity, this could be related to the low power of the statistical test used to assess such deviations (judged by a deterioration of the fit to the corresponding fMet-Leu-Phe self-competition curve [25]). The present study did, in fact, provide suggestive evidence that a model with a fixed proportion of high-affinity sites was not appropriate, in that the estimated proportion of these sites (geometric mean of 30%) in the competitive binding studies that included one of the most potent analogues (fMet-Leu-Phe-Phe or fMet-Leu-Phe-NHBzl) was greater than the estimate (13%) from studies that included only weaker analogues. Moreover, individual (unconstrained) analysis of binding data for fNle-Leu-Phe-Tyr suggested that this formyl peptide, the next most potent, might also bind to a greater proportion of high-affinity sites [12]. In conjunction with the anomalies in the biological responses, these results strongly favour a model of interconvertible affinity states over one with fixed states. Such a model can, moreover, explain the present observations (Fig. 6). Binding of a formyl peptide agonist to the receptor causes its conversion to a high-affinity state and triggers an immediate signal for degranulation; this biological response thus parallels high-affinity binding. When the agonist is a less potent formyl peptide (such as fMet-Leu-Phe), this activated state is maintained only for a short time; a rapid transition occurs to a lower-affinity state incapable of sustaining a chemotactic signal (Fig. 6a). This transition does not affect the degranulation signal, but restricts the longer-duration signal for the chemotactic response; migration is thus limited. The most potent analogues (such as fMet-Leu-Phe-Phe), in contrast, stabilize the activated high-affinity state in some way, thereby providing a longer duration signal and greater migration. As a result of its short duration, the signal from the less potent formyl peptides may, at relatively low receptor occupancy, saturate a subsequent step in the biochemical pathway leading to the chemotactic response. It is postulated, however, that such saturation does not occur with the signal of longer duration from the most potent analogues (Fig. 6a). Through the concept of spare receptors [51,52], such a pattern could explain the lower ED_{50} than K_d values for the less potent, but not the most potent, analogues. This conceptual model is thus in accord with all of the present data.

It is important to relate this conceptual model to the underlying biochemical processes. Other studies provide compelling evidence that a guanine-nucleotide-binding regulatory protein (G-protein)

transduces the formyl peptide signal from the receptor to the appropriate effector (presumed to be a phosphatidylinositol-specific phospholipase C) [2,4,7,11,40,53–55]. It is thus likely that interactions between receptor and G-protein account for the interconvertible affinity states. Current mechanisms for receptor–G-protein interactions [56–60] envisage a low-affinity receptor state (receptor alone; state I in the scheme of Fig. 6b) that undergoes a transition to a high-affinity state (receptor–G-protein complex; state II) upon agonist binding. A three-way fragmentation of the complex is then thought to occur (yielding state IV), separating the receptor, G_α and $G_{\beta\gamma}$ components; the released G_γ subunit interacts with the effector, causing cellular activation. This particular mechanism provides no scope for a ligand-dependent activated state. Thermodynamic considerations, however, argue against the three-way fragmentation step and imply that some form of intermediate (a complex of either receptor– $G_{\beta\gamma}$ or receptor– G_α) must occur [58]. The scheme in Fig. 6(b) incorporates such an intermediate (state III), and represents a feasible biochemical counterpart to the conceptual model (Fig. 6a). The receptor and its associated G-protein are considered to exist in four different states (Fig. 6b), two of high ligand-binding affinity (II and III). Binding of any agonist to the receptor drives the equilibrium from its resting state (a balance between states I and II, with state I predominant) towards the high-affinity activated state III. The most potent formyl peptides (fMet-Leu-Phe-Phe and fMet-Leu-Phe-NHBzl) temporarily stabilize and maintain this activated state, whereas the less potent analogues allow the reaction to proceed readily to a second, low-affinity, activated state (IV). State IV is regarded as transient; state III thus makes the dominant contribution to the overall activation and determines the signal for chemotaxis. Only the initial activation of the G-protein, however, is considered to determine the rapid signal for degranulation, its duration being limited by some subsequent process; this response is unaffected by the stabilization (or otherwise) of state III. This biochemical model thus incorporates the salient features of the conceptual model (Fig. 6a), particularly the idea that differential stabilization of the activated state III by different agonists may explain the increased chemotactic efficacy of the most potent formyl peptides.

The most important feature of this model is the concept of agonist-dependent stabilization of an activated receptor state (III). As the degree of stabilization of this state may depend on the particular ligand bound to the receptor, the model provides an explanation for the different properties of full and partial agonists. In addition, an antagonist can be regarded in this context as a ligand that binds to the receptor but completely destabilizes the activated state. These features of the model may have much broader applicability beyond the chemotactic formyl peptide receptors in the neutrophil.

There are several parallels between the model proposed in this study and the mechanism for neutrophil activation put forward by Sklar and his colleagues [2,17,41,48,61]. On the basis of competitive kinetic studies with a fluoresceinated ligand, the latter investigators have reported different residence times at the receptor for full agonists, partial agonists and antagonists, with the full agonists showing the longest residence [41]. Our proposals for the high-affinity activated state III promoted by full and partial agonists are closely analogous.

In conclusion, most chemotactic formyl peptides bind to their neutrophil receptors in a heterogeneous manner, but those of greatest potency show a homogeneous binding pattern. The relative potencies of a series of formyl peptide analogues for stimulation of degranulation correlate well with their potencies for high-affinity, but not low-affinity, binding. The complexity in the patterns for stimulation of chemotaxis cannot be explained by a model comprising two (or more) independent sets of

receptors of fixed affinity; instead, a model comprising interconvertible states of different affinities is required. The state of higher affinity appears to play a central role in initiating both degranulation and chemotaxis. Stabilization of this state by the most potent formyl peptide analogues can explain their greater efficacy in stimulating chemotaxis. This model may also apply to other receptors that are coupled by a G-protein to their associated effector. It can provide an explanation for differences in relative efficacy among different biological responses.

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