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A series of six ligands for the human formyl peptide receptor: Tetrapeptides with high chemotactic potency and efficacy

(fMet-Leu-Phe/monocyte/superoxide/ligand binding)

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ABSTRACT **We recently isolated, from culture fluids of *Staphylococcus aureus*, a chemotactic peptide that comprised equimolar quantities of methionine, leucine, phenylalanine, and isoleucine. It interacted with the formylmethionyl peptide receptor of human leukocytes and had considerably higher potency and efficacy than the widely studied tripeptide agonist fMet-Leu-Phe. On the assumption that the attractant was a formylmethionyl tetrapeptide, we synthesized the six possible sequences and tested the products for chemotactic potency and efficacy, as well as their capacity to inhibit binding of fluorescein isothiocyanate-labeled fMet-Leu-Phe-Lys to human monocytes. The concentrations required for inhibition of fluorescein-labeled fMet-Leu-Phe-Lys binding by the six peptides covered three orders of magnitude. Chemotactic potency (concentration that caused 50% of the maximum chemotactic response) ranged from 3.1×10^{-11} M to 6.4×10^{-10} M; efficacy (percentage of monocytes migrating at optimal attractant concentration) ranged from 41% to 66%. When the six synthetic tetrapeptides were ranked for chemotactic efficacy, they paired according to the position of phenylalanine. The average percentage migration was 66% for the two peptides with phenylalanine in position 3, 51% for phenylalanine in position 4, and 41% for phenylalanine in position 2. Since the published value for the percentage of human monocytes with detectable formyl peptide receptors is 60%, it is apparent that the two tetrapeptides with phenylalanine in position 3 (fMet-Ile-Phe-Leu and fMet-Leu-Phe-Ile) are full chemotactic agonists, which are capable of inducing migration of all the receptor-bearing cells. This is in contrast to the tripeptide fMet-Leu-Phe, which induces migration of only 50% of monocytes with receptors (efficacy of 33%). Since the chemotactic efficacy of the six tetrapeptides covers a wide range, the series may be useful to investigate signals that lead to directed movement after occupancy of receptors by chemoattractants.**

In 1975 Schiffmann and colleagues showed that among a series of synthetic formylmethionyl peptides several were chemoattractants for mammalian leukocytes (1). The most potent was formylmethionylleucylphenylalanine (fMet-Leu-Phe). It was suggested that formyl peptides were released by bacteria in the course of protein synthesis and that they induced a mammalian leukocyte response to prokaryotic invasion. The response resulted in not only chemotaxis but also a number of metabolic events, collectively termed the metabolic burst, which included hexose monophosphate shunt activation, release of oxygen radicals, and exocytosis of enzymes from cellular organelles. The availability of a defined agonist made the interaction of fMet-Leu-Phe with formyl peptide receptors a major model of leukocyte activation. The result has been more than a decade of studies of cell functional responses, biochemical changes, definition of second-signal responses to ligand–receptor interaction, receptor isolation, and agonist–receptor topographic modeling.

In the course of relating chemotaxis to a response to bacterial infection, we and others returned to the problem that stimulated Schiffmann's original research and attempted to isolate and characterize chemoattractants from bacterial culture fluid. Published reports include amino acid composition, but not molar ratios, of two peptides from *Streptococcus sanguis* cultures (2) and identification of fMet-Leu-Phe in a reverse-phase column fraction from *Escherichia coli* culture fluid (3).

From 70 liters of *Staphylococcus aureus* culture filtrate, we purified to apparent homogeneity 30 ng of a potent chemoattractant for human blood monocytes (A.R., L.E.H., R. Sowder, and E.J.L., unpublished results). The efficacy of this attractant (the percentage of monocytes responding to optimal chemoattractant concentration) was greater than that of fMet-Leu-Phe. The attractant comprised equimolar amounts of methionine, leucine, phenylalanine, and isoleucine. It inhibited binding of the labeled formylmethionyl chemoattractant fMet-Leu-Phe-Lys to human monocytes, which suggested that it was a formylmethionyl peptide. On the assumption that the attractant was a tetrapeptide, rather than a higher multiple of the four amino acids, we synthesized the six possible formylmethionyl peptide sequences and compared the products with respect to binding as well as potency and efficacy for both chemotaxis and superoxide release.

METHODS

Synthetic Peptides. Tetrapeptides were synthesized from preformed symmetric amino acid anhydrides on solid supports (4) in a model 430A Applied Biosystems peptide synthesizer (Applied Biosystems, Foster City, CA). Amino acids, protected at the α-amino group by a *tert*-butoxycarbonyl group (Boc) were from Applied Biosystems. Boc-Phe- and Boc-Ile-substituted resins were from Peninsula Laboratories (Belmont, CA); Boc-Leu-substituted resin was from Applied Biosystems. Details of peptide synthesis, deprotection, and characterization are published (5). In brief, peptides were severed from the Boc-protecting group and resin by reaction with hydrogen fluoride containing 10% anisole at 0°C for 1 hr in an all-Teflon apparatus. Each peptide was extracted into 50% acetic acid, diluted with water, and lyophilized. Peptides were purified by reverse-phase high-pressure liquid chromatography (HPLC) on a μBondapack C_{18} column (19 × 150 mm; Waters Associates) at pH 2.0 by

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Abbreviations: FITC, fluorescein isothiocyanate; Boc, *tert*-butoxycarbonyl.

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using 0–60% acetonitrile gradient elution (6). Composition of the A_{206} peptide peaks was confirmed by amino acid analysis. The α-amino group of each peptide was formylated by addition of acetic anhydride, over a period of 10 min, to a stirred solution of peptide in 98% formic acid at 4°C (7). After a total of 40 min, the reaction mixture was diluted with water and lyophilized. Formyl peptides were purified as above. Structure and amino acid sequence were confirmed by mass spectral and amino acid analyses.

Monocyte and Neutrophil Chemotaxis Assays. Mononuclear cells from human venous blood were separated by centrifugation on Ficoll/Hypaque (lymphocyte separation medium; Litton Bionetics) and used for chemotaxis assays in multiwell chambers (8). Cell suspensions were added to upper wells of the chambers; they were separated from lower wells containing chemoattractant by a 10-μm-thick polycarbonate membrane with 5-μm-diameter holes. The number of monocytes that migrated through the holes to the attractant side of the membrane during a 90-min incubation was counted with an image analyzer (9). The results were expressed as the percentage of the input number of monocytes that migrated per well (for duplicate wells). Neutrophils were separated by dextran sedimentation and assayed for chemotactic responses as previously described (10).

Binding of Fluorescein-Labeled fMet-Leu-Phe-Lys to Human Monocytes and Inhibition of Binding by Unlabeled Peptides. The formylmethionyl peptide chosen for fluorescein labeling was fMet-Leu-Phe-Lys. Its action as a chemoattractant is similar to that of fMet-Leu-Phe; the terminal lysine provides an amino group for binding of fluorescein isothiocyanate. Equilibration of monocytes with fluoresceinated ligand was done as described previously (11) with modifications to minimize the amount of peptide required. We determined the concentration of fMet-Leu-Phe-Lys–FITC needed to reach the plateau of the binding curve; this concentration was used in the assay for inhibition of binding by unlabeled peptide. Equal volumes (28 μl) of 8×10^{-7} M fMet-Leu-Phe-Lys–FITC at 0°C and dilutions of unlabeled peptide were mixed in 0.5 ml Eppendorf microfuge tubes (Brinkmann Instruments). Fifty microliters of these mixtures were transferred to ice-cold polypropylene tubes (2063; Falcon Plastics), and 50-μl aliquots of a mononuclear cell suspension containing 2×10^6 monocytes per ml were added. Tubes were shaken at 10-min intervals, and at the end of 30 min they were filled with medium (Gey's balanced salt solution/2% bovine serum albumin at 0°C) and centrifuged at 400 × *g* for 10 min. The supernatants were decanted; cells were washed again and suspended in 0.8 ml of Gey's solution/2% bovine serum albumin (0°C). Flow cytometry was done within 2 hr as described (11). Gating for monocytes was determined by the dot plot generated by forward angle versus right-angle light scatter. Fluorescence intensity of gated monocytes was recorded as a channel number over a linear range of 1–255 and analyzed on a Consort 40 computer. Data are presented as the mean channel number minus the value for cells equilibrated in medium without fluoresceinated peptide.

Assay of Superoxide Release. Superoxide release was measured as superoxide dismutase-inhibitable reduction of cytochrome *c* (12). For comparison of the dose–response curves of two or more peptides, serial dilutions of peptides were made in 0.9 ml of 0.1 mM cytochrome *c* in Hanks' balanced salt solution at 37°C. Fifty microliters of a mononuclear cell suspension, prepared as described for the chemotaxis assay and brought to a concentration of 5×10^6 monocytes per ml, was added to each reaction tube, and the tubes were incubated for 20 min. Controls included tubes without stimuli and tubes with peptide plus superoxide dismutase. After incubation, tubes were cooled in an ice bath and then centrifuged at 400 × *g*. The A_{550} of the supernatants was measured in a double-beam spectrophotometer with a superoxide dismutase control in the reference cuvette. Data were expressed as nanomoles of superoxide released per 10^7 monocytes, based on an extinction coefficient for cytochrome *c* reduction of 20 $mM^{-1} \cdot cm^{-1}$.

RESULTS

Competition for Binding Sites Between Tetrapeptides and Fluoresceinated fMet-Leu-Phe-Lys. To determine if the series of formyl tetrapeptides interacted with the human monocyte formyl peptide receptor, the peptides were tested for their capacity to inhibit binding of fMet-Leu-Phe-Lys–FITC at 0°C when the peptide was added simultaneously with the fluoresceinated ligand. The results of a comparison of all six tetrapeptides in a single experiment is shown in Fig. 1. Inhibition by the parent compound, fMet-Leu-Phe-Lys, is shown for reference. Each of the synthetic peptides inhibited binding of fMet-Leu-Phe-Lys–FITC. The slopes of the six inhibition curves are similar. The positions of the curves on the concentration axis cover a wide range on either side of the reference ligand. The concentration that caused 50% inhibition of fluoresceinated peptide binding (IC_{50}) was determined graphically, and the results from five experiments are summarized in Table 1. These values can be used to rank the six tetrapeptides according to their relative binding affinities, but they are not precise measures of agonist–receptor K_d values. The IC_{50} for fMet-Leu-Phe-Lys was 2.9×10^{-7} M, which is in reasonable agreement with the expected value of 2×10^{-7} M based on dilution of fluorescein-labeled peptide with unlabeled peptide. The IC_{50} values for the six tetrapeptides covered a range of three orders of magnitude.

Monocyte and Neutrophil Chemotactic Responses. Chemotactic responses of human monocytes from a single donor to the six tetrapeptides are shown in Fig. 2. The dose–response curves are typical of chemoattractants; they rise to a peak and then decline to zero as the concentration of ligand is increased above its optimum value. The most effective attractant caused migration of almost 70% of the monocytes added to assay wells, whereas only 35% migrated to the least effective peptide in the series. Among the six tetrapeptides, the concentration required for 50% of the maximal response

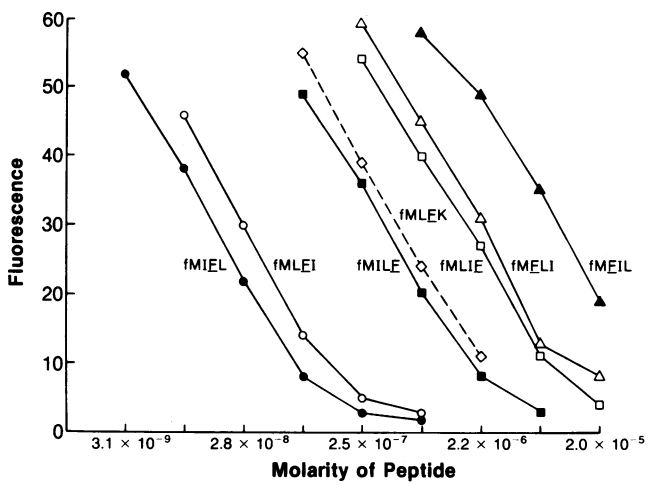

FIG. 1. Inhibition of binding of fMet-Leu-Phe-Lys–FITC to human monocytes by formylated tetrapeptides. Mean monocyte fluorescence (channel number) after subtraction of autofluorescence value versus molarity of unlabeled peptide (logarithmic scale). Concentration of peptide differs by a factor of 3 per scale division in Figs. 1–4. The abbreviations are based on the one-letter code for amino acids (f, formyl; F, phenylalanine; I, isoleucine; K, lysine; L, leucine; M, methionine).

Table 1. Summary of data on binding and chemotactic responses

Peptide	IC_{50}, M	Relative affinity	Efficacy	EC_{50}, M	Relative potency
fMIFL	$0.9 \pm 0.2 \times 10^{-8}$	1000	67 ± 4	$3.1 \pm 0.8 \times 10^{-11}$	100
fMLFI	$2.4 \pm 0.7 \times 10^{-8}$	375	65 ± 2	$3.4 \pm 0.5 \times 10^{-11}$	91
fMILF	$2.4 \pm 0.7 \times 10^{-7}$	38	52 ± 2	$9.6 \pm 2 \times 10^{-11}$	32
fMLIF	$9.0 \pm 2.0 \times 10^{-7}$	10	50 ± 1	$1.2 \pm 0.3 \times 10^{-10}$	26
fMFLI	$1.0 \pm 0.3 \times 10^{-6}$	9	41 ± 3	$3.6 \pm 1.5 \times 10^{-10}$	9
fMFIL	$6.1 \pm 2.0 \times 10^{-6}$	1.5	41 ± 1	$6.4 \pm 2.4 \times 10^{-10}$	5
fMLFK	$2.9 \pm 0.2 \times 10^{-7}$	31	35 ± 2	$2.7 \pm 1.1 \times 10^{-10}$	11
fMLF			36 ± 0	$1.0 \pm 0.5 \times 10^{-9}$	3

IC_{50} (measure of binding affinity), concentration (molar) that caused 50% inhibition of fMLFK-FITC binding. Values are given as the mean ± SEM for five experiments. Relative affinity, (IC_{50} of fMFIL/IC_{50} of peptide) × 1.5. Efficacy, percentage of monocytes migrating at the optimal peptide concentration. EC_{50} (measure of chemotactic potency), concentration (molar) that induced 50% of the maximal chemotactic response. Relative potency, (EC_{50} of fMLF/EC_{50} of peptide) × 3.2. There is a linear relationship between efficacy and potency among the six tetrapeptides, with a correlation coefficient of 0.99. For the regression line of efficacy versus relative binding affinity, the correlation coefficient is 0.84. Data for the efficacy, EC_{50}, and relative potency are based on four experiments with the first six tetrapeptides and on two experiments with fMLF and fMLFK. Abbreviations are as in Fig. 1.

(EC_{50}) covered a 20-fold range, from 2.1×10^{-11} M to 4.6×10^{-10} M. Similar results were obtained with neutrophils from the same donor (Fig. 3).

Efficacy and potency for monocyte chemotaxis are shown for fMet-Leu-Phe, fMet-Leu-Phe-Lys, and the six tetrapeptides (Table 1). Chemotactic efficacy of fMet-Leu-Phe and fMet-Leu-Phe-Lys is less than the lowest of the tetrapeptide series and confirms published values of 33% and 36%, respectively (11). The six tetrapeptides are grouped in pairs according to the position of phenylalanine. The pairs follow the same order for chemotactic efficacy and potency: the order for effectiveness is phenylalanine in position 2 < phenylalanine in position 4 < phenylalanine in position 3. For the two members of a pair with a given phenylalanine position, chemotactic efficacy (percentage of cells migrating at optimal attractant concentration) is the same and is unaffected by the positions of leucine and isoleucine. The mean efficacy values for the three pairs are 66%, 51%, and 41% (Table 1) for phenylalanine in position 3, position 4, and position 2, respectively.

Tetrapeptide-Induced Monocyte Superoxide Release. The metabolic effects of ligand interaction with the formyl peptide receptor of human monocytes include production and release of superoxide anion (13), which is mediated by activation of NADPH oxidase. Release was monitored by addition of cytochrome *c* to the assay medium and measurement of its reduction by superoxide during an incubation of 20 min. Fig. 4 is an example of results obtained in an assay of high- and low-potency members of the six tetrapeptide series. The molarity required for 50% of the maximal endpoint response differed by a factor of ≈50 for these two peptides, which reflects potency differences observed for binding and chemotaxis. In contrast to the efficacy differences for chemotaxis, the amount of superoxide released at optimal concentrations of the high- and low-potency peptides did not differ.

DISCUSSION

In a previous study we reported that only one-third of all human monocytes migrated in chemotaxis chambers to optimal concentrations of fMet-Leu-Phe or fMet-Leu-Phe-Lys (11). Analysis by flow cytometry of fMet-Leu-Phe-Lys–FITC binding at 0°C showed that 60% of human monocytes have detectable formyl peptide receptors (11). Therefore, fMet-Leu-Phe or fMet-Leu-Phe-Lys, at optimal concentrations, induced migration of only about 50% of the monocytes with formyl peptide receptors (about 30% migrating compared to 60% with receptors). In contrast, efficacy data in Table 1 show that an average of 66% of human monocytes migrated in response to the synthetic tetrapep-

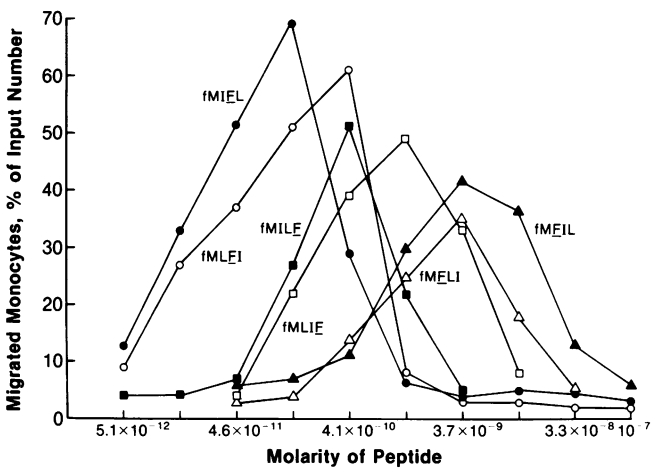

FIG. 2. Chemotactic responses of monocytes from a single donor to the six members of the formyl tetrapeptide series. Serial 3-fold dilutions of fMet-Leu-Phe and fMet-Leu-Phe-Lys were assayed at the same time; peak responses were 36% and 35%, respectively. Abbreviations are as in Fig. 1.

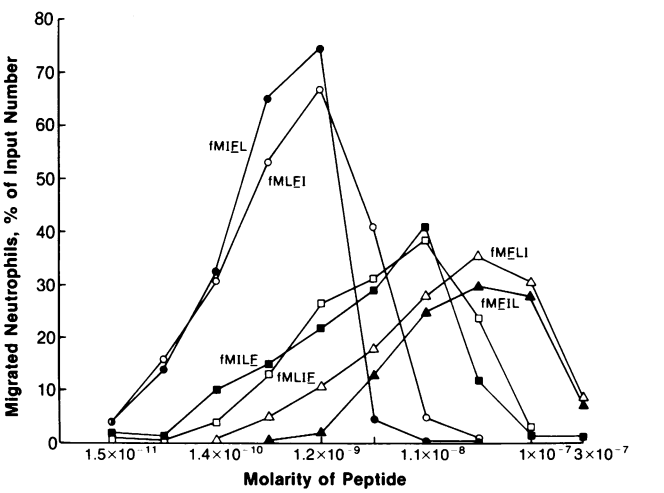

FIG. 3. Chemotactic responses of neutrophils from a single donor to the six members of the formyl tetrapeptide series. Peak responses to simultaneously assayed fMet-Leu-Phe and fMet-Leu-Phe-Lys were 30% and 31%, respectively. Abbreviations are as in Fig. 1.

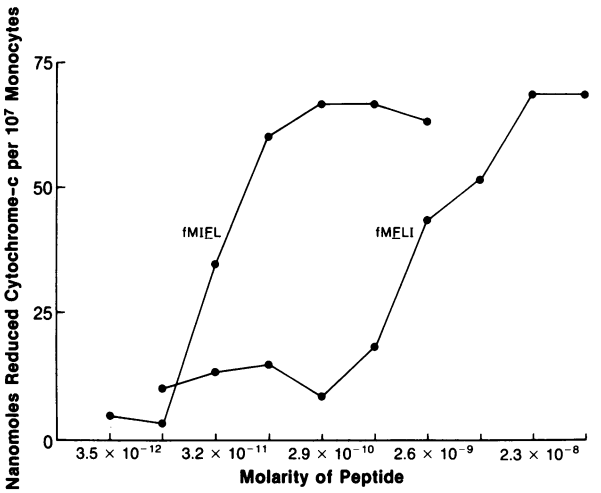

FIG. 4. Dose–response curves for superoxide release by human monocytes over a period of 20 min of incubation with fMet-Ile-Phe-Leu or fMet-Phe-Leu-Ile. Abbreviations are as in Fig. 1.

tides fMet-Ile-Phe-Leu or fMet-Leu-Phe-Ile. Since this represents migration of all receptor-bearing monocytes, these two peptides function as full agonists for induction of human monocyte chemotaxis. The data suggest that if an agonist makes a sufficiently good fit with the monocyte formyl peptide receptor, all the receptor-bearing monocytes respond with directional movement.

Since the studies of Schiffmann *et al.* (1) on synthetic chemotactic peptides, there has been a series of papers, representing the collaborative effects of three laboratories, on structural requirements for formyl peptide agonists (14–17). Although the chemotactic response to agonists was measured in two publications (14, 15), the first paper in the series stated that no differences in chemotactic efficacy were found (14), and no reference to efficacy was made in the subsequent publication (15). Comparisons of the synthetic formyl peptides were made by determination of agonist concentration required for maximal lysozyme release (agonist potency). Structural requirements for optimal potency included N-terminal formyl substitution (in contrast to acetyl, for example), methionine in position 1, phenylalanine in position 3, and a nonpolar amino acid in position 4 (14–17). As an example of the position 4 requirement, fMet-Leu-Phe-Ile was 10 times more potent than fMet-Leu-Phe for lysozyme release (16). Our data on potency of these two peptides for chemotaxis are comparable (Table 1); i.e., the relative potency ratio of fMet-Leu-Phe-Ile to fMet-Leu-Phe is 30. In our tetrapeptide series, we also confirm the importance of phenylalanine in position 3 for chemotaxis; the order for increasing potency is phenylalanine in position 2 < phenylalanine in position 4 < phenylalanine in position 3. Although the formyl peptide agonists studied by Freer *et al.* (16) differed in potency for lysozyme release, at their optimal concentrations no difference in efficacy (quantity of lysozyme released) was observed, perhaps because the response was close to maximal. Likewise, among the synthetic tetrapeptides in our study, we observed no differences in efficacy for superoxide release (Fig. 4).

In contrast, striking differences in chemotactic efficacy were found in the tetrapeptide series. The most significant findings of this investigation are the high efficacy of the tetrapeptides compared to the reference tripeptide, fMet-Leu-Phe, and the ordered difference in chemotactic efficacy among the six tetrapeptides. The order follows the same order as the relative potency, confirms the importance of phenylalanine in position 3, and indicates the interchangeability of leucine and isoleucine in positions 2 and 4 (Table 1).

Niedel *et al.* (18), in a study on human neutrophils, found a correlation between binding affinity and chemotactic potency for five different formyl peptides ranging in length from 3 to 6 residues. Although the percentage of cells migrating was not reported, a figure in their paper shows that the chemotactic response to the tripeptide fMet-Leu-Phe was lower than that of the longer peptides.

The data on chemotactic efficacy suggest that agonist–receptor fit may be more critical for chemotaxis than for metabolic response or granule release. Receptor occupancy by either low- or high-potency peptides at optimal concentration induces the same magnitude of metabolic response. In contrast, receptor occupancy by high- or low-potency agonists results in different chemotactic efficacy. Freer *et al.* (16) suggested that there are multisite interactions between the formyl peptide and its receptors. Are there separate receptor domains, activation of which leads to different second signals that initiate the different pathways for movement and metabolic responses, or does agonist–receptor interaction cause release of only one second signal that stimulates the different pathways? The series of six tetrapeptides may be useful in investigating signals that generate the chemotactic response because they provide the opportunity to correlate chemotactic efficacy with stimulus-induced changes in transductional pathway intermediates.

Our original purpose in synthesizing the formylmethionyl tetrapeptides was to deduce a probable structure of the pure tetrapeptide isolated from *S. aureus* culture fluid. We had established the amino acid composition of the purified peptide, but we had insufficient material to obtain the sequence. We therefore determined if the activity of the bacterial peptide corresponded to any of the six possible sequences of the synthetic formylmethionyl tetrapeptides. Chemotactic efficacy of the bacterial peptide (52 ± 4%; mean ± SEM for six determinations) was comparable to only two of the six synthetic peptides. These were fMet-Ile-Leu-Phe and fMet-Leu-Ile-Phe, with chemotactic efficacies of 52% and 50%, respectively (Table 1). Both the bacterial peptide (A.R., L.E.H., R. Sowder, and E.J.L., unpublished results) and fMet-Ile-Leu-Phe (Fig. 1) inhibited fMet-Leu-Phe-Lys–FITC binding with dose–response curves comparable to that of the unlabeled peptide fMet-Leu-Phe-Lys. It is likely, therefore, that the bacterial peptide is fMet-Ile-Leu-Phe, since it is the only synthetic tetrapeptide with both chemotactic efficacy and relative binding affinity corresponding to that of the natural product. It remains to be seen if the synthetic peptides fMet-Leu-Phe-Ile and fMet-Ile-Phe-Leu, with efficacies of 65% and 67%, respectively, are also bacterial products. Efficacy of all nine peaks of chemotactic activity obtained from reverse-phase HPLC of *S. aureus* culture fluid was greater than 60% (19). It is likely, therefore, that in contrast to the relatively low efficacy of fMet-Leu-Phe, a number of chemotactic peptides produced by bacteria are capable of inducing migration of all receptor-bearing monocytes.

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