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Characterization of a class of nonformylated *Enterococcus faecalis*-derived neutrophil chemotactic peptides: The sex pheromones

(formyl peptide receptor/signal peptides/fMet-Leu-Phe/structure–activity studies)

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ABSTRACT **Bacteria produce a heterogeneous mixture of neutrophil chemotactic agents in culture filtrates. Formylmethionyl peptides have been shown to comprise a significant portion of the chemotactic activity in bacterial culture filtrates; however, not all of the chemotactic agents in bacterial culture filtrates are formylated peptides. To examine whether nonformylated peptides derived from bacteria could act as chemotactic agents, we studied several nonformylated hepta- and octapeptide *Enterococcus faecalis*-derived sex pheromones, their modified derivatives, and their competitive inhibitors for activation of rat peritoneal neutrophils. Several of these peptides, in particular cAM373 and cPD1, proved to be potent chemotactic agents in submicromolar concentrations as well as inducers of lysosomal granule enzyme secretion. Moreover, the more biologically active peptides were able to compete with fMet-Leu-[^{3}H]Phe for binding to the formyl peptide receptor. These studies demonstrate that the formylmethionyl moiety may be an absolute requirement only for the binding of di- and tripeptides to the formyl peptide receptor. Larger peptides that may have or that may allow for additional contact points between the peptide and receptor may require N-formylation only relatively. Indeed, by removing this structural restraint, the formyl peptide receptor may interact with an unlimited number of peptide fragments of both infectious and host origins to then modulate neutrophil responses to infection and inflammation.**

Most bacteria elaborate chemotactic factors in culture filtrates (1, 2). During the inflammatory response associated with bacterial infections, neutrophils migrate into tissues containing viable bacteria and participate in their elimination (by phagocytosis) from the tissue through a number of metabolic events, collectively termed the metabolic burst, which include hexose monophosphate shunt activation, release of oxygen free radicals, and lysosomal granule enzyme secretion (3, 4). These events are initiated by the release from bacteria of chemotactic factors as well as by the local activation of the complement cascade (5, 6). In addition, this process may be further amplified by the release from damaged host cell membranes of lipid and protein constituents that have chemotactic activity (7, 8).

A specific class of prokaryotic cell products, namely the formylmethionyl peptides derived from the NH_2-terminal regions of newly synthesized proteins, have been thought to be the major chemotactic factors in bacterial culture filtrates (9). However, detailed structural analysis of the bacterial chemotactic substances has been hampered by their very low concentrations (5, 10, 11). Although early studies described the presence in *Escherichia coli* culture filtrates of chemotactic activities soluble in chloroform/methanol with properties of a fatty acid (12, 13), structural analysis of this material has not been pursued. We have shown (14) that *E. coli* culture filtrates contain at least five chemotactic peptides of which, by radioimmunoassay, three appear to be formylated peptides. One of these peptides was determined to be fMet-Leu-Phe by gas chromatography/mass spectroscopy analysis. However, at least two of these five activities may have been structurally unrelated to the formyl peptides (14).

In our search to provide direct evidence that bacteria-derived nonformylmethionyl peptides have chemotactic activity, we examined peptides cPD1, cAD1, and cAM373, which are sex pheromones with seven or eight amino acid residues excreted by recipient (plasmid-free) strains of *Enterococcus faecalis* (formerly *Streptococcus faecalis*). These sex pheromones are found in significantly higher concentrations in culture filtrates (cAD1 and cPD1; 1–3 × 10^{-9} M) and (cAM373; 2–4 × 10^{-9} M) (15, 16) when compared to fMet-Leu-Phe; 6–7 × 10^{-10} M (14). Donor strains, harboring the conjugative plasmid pPD1, pAD1, or pAM373, respectively, respond to the pheromone by synthesizing a surface adhesin that facilitates formation of mating aggregates. Plasmids pPD1 and pAD1 encode peptides iPD1 and iAD1, which act as competitive inhibitors of cPD1 and cAD1, respectively (for review, see refs. 16 and 17). Our experiments demonstrate that these nonformylated peptides are potent chemotactic factors and inducers of lysosomal granule enzyme secretion for rat peritoneal neutrophils. In addition, our studies show that these nonformylated peptides bind to the formyl peptide receptor and have much greater biological efficacies than expected. The significance of these findings as they relate to peptide–receptor interactions are discussed.

MATERIALS AND METHODS

Sex Pheromones. The nomenclature of the peptides used in these studies and their amino acid sequences are as follows: fMet-Leu-Phe, where f is formyl; cAM373, H-Ala-Ile-Phe-Ile-Leu-Ala-Ser-OH (18); cPD1, H-Phe-Leu-Val-Met-Phe-Leu-Ser-Gly-OH (19); cPD1N, H-Phe-Leu-Val-Met-Phe-Leu-Ser-NH_2; iPD1, H-Ala-Leu-Ile-Leu-Thr-Leu-Val-Ser-OH (20); cAD1, H-Leu-Phe-Ser-Leu-Val-Leu-Ala-Gly-OH (21); cAD1N, H-Leu-Phe-Ser-Leu-Val-Leu-Ala-NH_2; and iAD1, H-Leu-Phe-Val-Val-Thr-Leu-Val-Gly-OH (22). The peptides cPD1N and cAD1N are synthetic derivatives where the COOH-terminal amino acid residue (glycine) of cPD1 and cAD1, respectively, is absent, and an amide terminus is now present. All substances are soluble at concentrations up to 1 mg/ml in an aqueous solution of 50% (vol/vol) acetonitrile

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(HPLC grade) and 2% (vol/vol) trifluoroacetic acid (99.8% pure). The peptides remain in solution upon further dilution with Hanks' buffer (3). Synthetic forms of all of the peptides above have been prepared and cAM373, cPD1, iPD1, cAD1, and iAD1 are biologically active as enterococcal sex pheromones or inhibitors at concentrations as low as 5×10^{-11} M (15, 16).

Cells. Young male Long Evans rats were injected with 30 ml of sterile 0.1% oyster glycogen in 0.15 M NaCl and sacrificed 18 hr later with ether. The peritoneal cavities were opened surgically and the cells were obtained by repeated lavage with Hanks' buffer containing sodium heparin (1 unit/ml). For all experiments, cells from three or four rats were pooled. Morphologic analysis showed >85% polymorphonuclear leukocytes as determined by differential Wright's stain. Final cell suspensions contained 1% crystalline bovine serum albumin in Hanks' buffer.

Chemotaxis. The chemotaxis assay was carried out using modified Boyden chambers (23, 24). Briefly, fMet-Leu-Phe (Sigma) or test agents (pheromones) (dissolved in Hanks' buffer) were added to the lower compartment of a chemotaxis chamber. Rat peritoneal neutrophils were suspended in Hanks' buffer/1% bovine serum albumin (Sigma) at 1.5×10^6 cells per ml and added to the upper compartment of the chamber. The two compartments were separated from each other by a cellulose nitrate filter (5 μm, average pore size; Schleicher & Schuell) that had been soaked in Hanks' buffer/1% bovine serum albumin. For measurements of random migration, Hanks' buffer only was added to the lower compartment. For determination of chemokinetic activity, equal concentrations of chemotactic peptide or test agent (pheromone) were placed in the lower and the upper compartment (25). After incubation of the chemotaxis chamber in humidified air for 60 min at 37°C, the filter was removed, stained with hematoxylin, dried in isopropanol, and cleared with xylene. The distance of migration of the neutrophils within the filter was measured under light microscopy by the "leading front" method (25).

Lysosomal Enzyme Release. The lysosomal enzyme release assay was performed with 5×10^6 cells per ml in 5 μg of cytochalasin B (Aldrich) and was terminated after 5 min at 37°C by immersing the tubes in an ice bath and then centrifuging. β-Glucuronidase activity was assayed in the supernatant using phenolphthalein glucuronidate as a substrate (26).

Inhibition of fMet-Leu-[^{3}H]Phe Binding. To determine if the sex pheromones bound to the rat neutrophil formyl peptide receptor, inhibition of fMet-Leu-[^{3}H]Phe binding was investigated. All binding studies were carried out at 24°C in Hanks' buffer supplemented with 1.6 mM calcium and 10 mM sodium azide at 2.5×10^7 cells per ml. Cells were always preincubated at 24°C for 30 min before initiation of a binding study at the same temperature. The binding studies were performed by incubating 100 μl of cells, 1.0 pmol of fMet-Leu-[^{3}H]Phe (47.6 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and 1:3 dilutions of the various sex pheromones in a 12 × 75 mm glass test tube. After 20 min, the cells were harvested by a glass fiber vacuum filtration method and analyzed for cell-bound radioactivity (24). Nonspecific binding was defined as the amount of fMet-Leu-[^{3}H]Phe bound in the presence of a 1000-fold molar excess of nonradiolabeled fMet-Leu-Phe (Sigma) and was always <10% of total binding.

RESULTS

Fig. 1 shows the chemotactic activity of the various sex pheromones, their modified derivatives, and their competitive inhibitors as compared with the formylated tripeptide fMet-Leu-Phe, when rat polymorphonuclear leukocytes (neutrophils) were used in the assays. The range of doses employed allowed the observation of the cell migratory

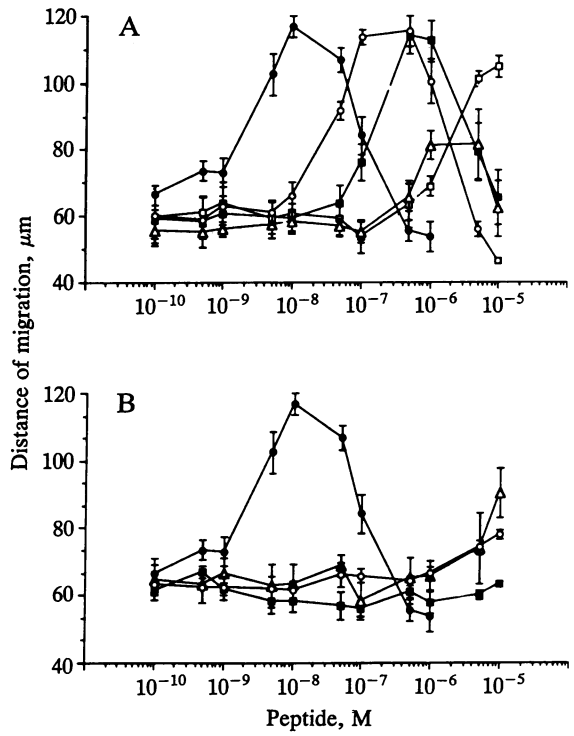

FIG. 1. Chemotactic activity of various sex pheromones. Tests were performed in triplicate. Cell migration was determined at five sites per filter on three filters and the mean ± SEM of three to five measurements was calculated and expressed in μm. In Hanks' buffer, control migration was 50.4 ± 7.2 μm. (*A*) Chemotactic activity of fMet-Leu-Phe (●), cAM373 (○), cDP1 (■), cPD1N (□), and iPD1 (△) on rat peritoneal neutrophils is shown. (*B*) Chemotactic activity of fMet-Leu-Phe (●), cAD1 (○), cAD1N (■), and iAD1 (△) is shown.

responses over a wide concentration gradient of the peptides. Two of the sex pheromones, cAM373 and cPD1, evoked equivalent maximal responses but exhibited reduced potencies relative to fMet-Leu-Phe (Fig. 1*A*). The mean EC_{50} values calculated from at least four experiments were $1.8 \pm 0.2 \times 10^{-7}$ M, $4.9 \pm 0.1 \times 10^{-8}$ M, and $3.4 \pm 0.3 \times 10^{-9}$ M for cPD1, cAM373, and fMet-Leu-Phe, respectively. The chemotactic activity of cPD1 markedly decreased by removing the COOH-terminal amino acid residue and amidating the peptide, to produce cPD1N. This was indicated by the displacement to the right of the corresponding concentration–response curve relative to cPD1 (Fig. 1*A*). The mean EC_{50} value calculated for cPD1N was $2.2 \pm 0.3 \times 10^{-6}$ M. The competitive inhibitor peptide iPD1 was active only at higher concentrations when compared to cPD1 and the mean EC_{50} value was $4.5 \pm 1.0 \times 10^{-7}$ M. In addition, this peptide appeared to be a partial agonist since a submaximal plateau of activity was obtained. The structurally unrelated sex pheromone cAD1 was a poor chemotactic factor with activity only at high concentrations $>5 \times 10^{-6}$ M, and similarly to the cPD1 peptides, removal of the COOH-terminal amino acid and amidation to produce cAD1N caused marked loss of activity (Fig. 1*B*). The inhibitor peptide iAD1 was the most active of these three peptides but had a high mean EC_{50} value of 1.7×10^{-6} M. The solvent employed to solubilize the materials produced no effect on the migratory behavior of the cells (data not shown).

To show that the responses into the filter resulted from a chemotactic response and not a chemokinetic response where increased random migration could result in greater penetration of cells into the filter, comparison of the experimental values obtained with the theoretical values for the predicted migration when the only variable is the rate of

random locomotion was performed. Results are presented in Table 1. Values in the table are the experimentally determined distances into the filter that the front two cells of the population moved when various concentrations of the peptides were placed above and below filter. Values in parentheses are the predicted distances the front two cells would move in a random walk. Accordingly, cAM373, cPD1, iPD1, and iAD1 behaved as true chemotactic agents, despite differences in activity. In addition, cAM373 stimulated random movement (chemokinesis) of the cells at concentrations up to 5×10^{-8} M, as indicated by penetration obtained when no gradient was present (values in boldface type in Table 1).

The effect of the competitive antagonist of the formyl peptide receptor Boc-Phe-Leu-Phe-Leu-Phe (Boc = butyloxycarbonyl; Peninsula Laboratories) on the chemotactic activities of cAM373 and cPD1 was studied. Concentrations of cAM373 and cPD1 giving 90% (EC_{90}) of their maximal chemotactic activity on rat peritoneal neutrophils were used and assays were performed in the absence or presence of 1×10^{-5} M Boc-Phe-Leu-Phe-Leu-Phe. The results calculated from the means of two experiments showed a statistically significant ($P < 0.05$) inhibition in the distance of migration (μm) for both the EC_{90} values of cAM373 (112 ± 3.4 vs. 98.0 ± 5.3) and cPD1 (106.5 ± 4.8 vs. 77.0 ± 3.3) (mean ± SEM, $n = 10$). These findings suggested that the latter peptides might interact with the formyl peptide receptor to evoke a migratory response of rat polymorphonuclear leukocytes.

Granule enzyme secretion was also induced by two of the more potent pheromones cAM373 and cPD1 (Fig. 2 and Table 2). As expected, the concentrations of the pheromones required to induce β-glucuronidase were greater than for chemotaxis (Table 2) (27). However, unlike in the chemotaxis assays where equivalent maximal response for fMet-Leu-Phe, cAM373, and cPD1 occurred (Fig. 1*A*), the two pheromones caused a submaximal release of β-glucuronidase (Fig. 2).

We next investigated the ability of the various pheromones, their modified derivatives, or their competitive inhibitors to bind to the formyl peptide receptor. As shown in Fig. 3 and Table 2, all peptides competed with fMet-Leu-[^{3}H]Phe, albeit with various degrees of binding efficiency. The most potent pheromone was again cAM373. Interestingly, peptides cPD1 and cPD1N had identical binding inhibition profiles despite the fact that cPD1N had reduced chemotactic activity (Fig. 1*A*) and was inactive in the enzyme release assay (Table 2) compared to the parent compound. The other peptides tested had activity only at higher concentrations.

DISCUSSION

Five critical areas of formyl peptide and receptor interaction have been defined through extensive peptide mapping studies

Table 1. Migration of neutrophils induced by several pheromones in the Boyden chamber assay

Pheromone	Migration, μm					
cAM373	0	5×10^{-8}	1×10^{-7}	5×10^{-7}	1×10^{-6}	5×10^{-6}
0	**58**	86 (66)	114 (76)	103 (81)	81	49
5×10^{-8}	70	**90**	104 (88)	111 (87)	82	54
1×10^{-7}	69	79	**86**	98 (80)	68	52
5×10^{-7}	60	66	70	**68**	68	54 (62)
1×10^{-6}	54	60	58	58	**55**	45 (53)
5×10^{-6}	40	43	48	39	42	**45**
cPD1	0	1×10^{-7}	5×10^{-7}	1×10^{-6}	5×10^{-6}	1×10^{-5}
0	**70**	88 (71)	120 (72)	118 (73)	76	82
1×10^{-7}	59	**74**	116 (74)	102 (74)	81	74
5×10^{-7}	48	62	**75**	72 (71)	66	71
1×10^{-6}	50	55	56	**62**	55	60
5×10^{-6}	32	35	29	32	**34**	41 (33)
1×10^{-5}	25	30	26	25	31	**26**
iPD1	0	1×10^{-7}	5×10^{-7}	1×10^{-6}	5×10^{-6}	1×10^{-5}
0	**51**	48	54 (51)	74 (52)	70	62
1×10^{-7}	50	**50**	55	65	71	51
5×10^{-7}	52	52	**52**	68	70 (59)	58
1×10^{-6}	48	52	57	**68**	62	55
5×10^{-6}	50	48	44	52	**48**	37 (45)
1×10^{-5}	31	38	44	36	38	**33**
iAD1	0	1×10^{-7}	5×10^{-7}	1×10^{-6}	5×10^{-6}	1×10^{-5}
0	**42**	36	44	48	46	74 (45)
1×10^{-7}	42	**40**	42	41	38	72
5×10^{-7}	35	31	**42**	43	58	70
1×10^{-6}	39	43	44	**43**	46	67
5×10^{-6}	36	42	48	46	**44**	59
1×10^{-5}	32	38	42	44	38	**44**

Values across the top of the matrices are molar concentrations of the pheromone below the filter; values along the left side of the matrices are molar concentrations of the pheromone above the filter. Migration is the measured mean distance from the top of the filter to the front of two polymorphonuclear leukocytes in one plane of focus (40× objective). Values in boldface type are the measured mean distance in a uniform concentration of pheromone and were used to calculate the distances to the front two cells of a hypothetical population undergoing purely random migration (values in parentheses). The cell migration was determined at five sites per filter on six filters and each data point represents the mean of 30 readings with SEM consistently ≤10%.

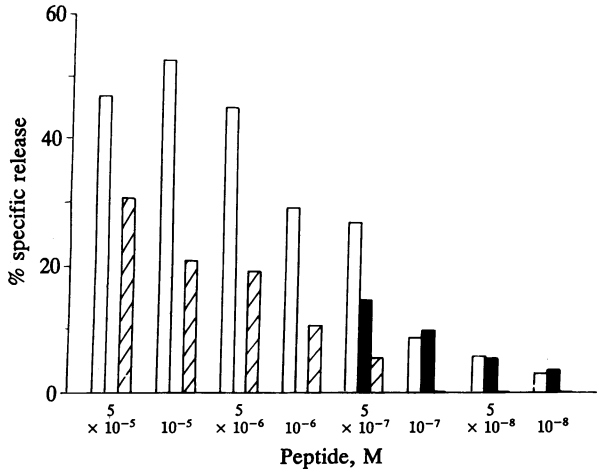

FIG. 2. Lysosomal enzyme releasing activity of cAM373 (solid bars), cPD1 (hatched bars), and fMet-Leu-Phe (open bars). Results show the specific release of β-glucuronidase expressed as percentage of specific release. Data are the mean of triplicate determinations of one representative experiment.

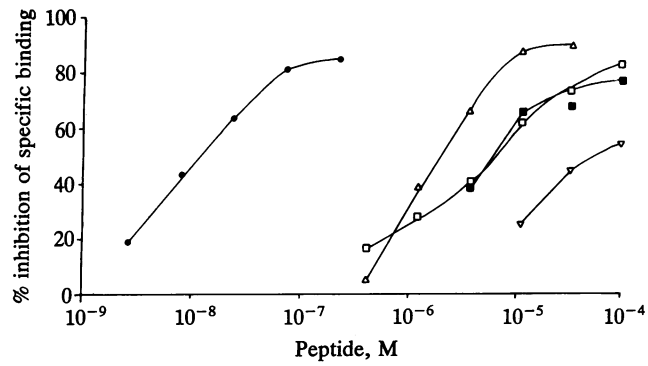

FIG. 3. Binding inhibition of fMet-Leu-[^{3}H]Phe. All data points were obtained in triplicate, and the SEM was consistently less than ±5%. Specific binding was calculated and is expressed as the percentage of inhibition of fMet-Leu-[^{3}H]Phe binding. ●, fMet-Leu-Phe; △, cAM373; ■, cPD1; □, cPD1N; and ▽, iAD1.

(27–31). Of importance here is the hydrophobic pocket in peptide position 2 that accommodates isoleucine (cAM373) and leucine (cPD1 and cPD1N) and to a lesser extent the leucine of iDP1. The relative lack of activity of the cAD1, cAD1N, and iAD1 peptides may be due to the adverse effects of phenylalanine in peptide position 2, as shown by prior studies in which there was a 350 times decrease in activity for Met-Phe-Leu compared to Met-Leu-Phe (27) and similar losses in activity in a series of phenylalanine-containing formylated tetrapeptides (32). In contrast, when phenylalanine is in peptide position 3, marked increases in activity have been seen (27, 32) and may explain the greater activity of cAM373 compared to the cPD1-related peptides. Likewise, phenylalanine in peptide position 1 is more active than alanine and may explain the greater activity of cPD1 compared to iPD1 (29, 30).

Of interest are the different biological efficacies of cPD1 and cPD1N despite similar binding affinities (Table 2). Since the formyl peptide receptor has been thought to optimally accommodate a tetra- to pentapeptide (30, 31, 33), effects of peptide amino acid substitutions beyond the fifth amino acid are quite surprising and raise the possibility of a larger binding site that may have or that may allow for additional contact points between the COOH terminus of the peptide and the receptor and that, in turn, may modulate the biological activity of the peptides. To our knowledge, only two studies (30, 33) have analyzed effects at the COOH end of the receptor site. The first study did so with two COOH-terminal-modified formylated tetrapeptides and showed that their benzyl ester derivatives were less active than the corresponding free acids (30), whereas the second study with three series of N^{α}-formylated homoligopeptides, from the dipeptide to the heptapeptide, derived from L-methionine, L-norleucine, and *S*-methyl-L-cysteine failed to show any change in activity beyond the tetrapeptide to pentapeptide stage (33).

It is also evident when comparing the chemotactic activity of the sex pheromones with their ability to inhibit fMet-Leu-[^{3}H]Phe binding that two peptides have more biologic activity than expected by their binding affinity. This can be seen in Table 2 when comparing cAM373 and cPD1, which are only 14 and 53 times less active, respectively, than fMet-Leu-Phe for chemotaxis but are 282 and 700 times less active, respectively, in their ability to inhibit fMet-Leu-[^{3}H]Phe binding. This is similar to the discrepancy (namely, an increase in the biological activity over binding affinity) that we have seen previously in a series of COOH-modified formylated tripeptides (benzyl esters) when compared to the corresponding free acids (30) but is in contrast to our early mapping studies with di- and triformyl peptides, which showed excellent correlations between binding affinity and biological efficacy (27–31). One might speculate that the increase in chemotactic activity is due to (*i*) binding to the high-affinity binding site not detected by using our standard binding conditions or (*ii*) the efficacy of these peptides is such that only a small portion of the total receptor population is necessary for full biological effectiveness (30, 34).

Table 2. Activity of various sex pheromones with rat neutrophils

Peptide	Amino acid sequence	ED_{50}, M		ID_{50}, M
		Chemotaxis	Enzyme secretion	
fMLP	fMLF	$3.4 \pm 0.3 \times 10^{-9}$ (4)	$2.53 \pm 1.0 \times 10^{-7}$ (2)	$7.97 \pm 3.5 \times 10^{-9}$ (4)
cAM373	AIFILAS	$4.9 \pm 0.1 \times 10^{-8}$ (4)	$1.18 \pm 0.4 \times 10^{-6}$ (2)	$2.25 \pm 0.8 \times 10^{-6}$ (2)
cPD1	FLVMFLSG	$1.8 \pm 0.2 \times 10^{-7}$ (4)	$2.52 \pm 1.4 \times 10^{-5}$ (3)	$5.60 \pm 0.8 \times 10^{-6}$ (3)
cPD1N	FLVMFLS-NH_2	$2.2 \pm 0.3 \times 10^{-6}$ (4)	—*	$5.00 \pm 0.5 \times 10^{-6}$ (2)
iPD1	ALILTLVS	$4.5 \pm 1.0 \times 10^{-7}$ (4)	—†	$>10^{-4}$ (2)
cAD1	LFSLVLAG	$>10^{-5}$	—*	$>10^{-4}$ (2)
cAD1N	LFSLVLA-NH_2	—*	—*	$>10^{-4}$ (2)
iAD1	LFVVTLVG	$1.7 \pm 0.6 \times 10^{-6}$ (4)	—*	$3.0 \pm 0.01 \times 10^{-5}$ (2)

ED_{50} is the concentration of peptide required to give 50% of the peptides maximal induced enzyme secretion or cell migration into the filter. Data are mean ± SEM. Values in parentheses are the number of experiments. ID_{50} is the concentration of peptide required to displace 50% of the specific fMet-Leu-[^{3}H]Phe binding. Data are mean ± SEM. Values in parentheses are the number of experiments. The single-letter amino acid code is used.

*Inactive at 10^{-5} M.

†Inactive at 5×10^{-6} M.

The formyl group has been long believed to be absolutely necessary for binding to the receptor. While this is true for smaller di- and tripeptides, it may not be true for larger peptides that are more stable in secondary structure and that have interactions with yet unknown parts of the binding site. Thus, although the formylmethionyl moiety is critical in some cases, our finding that peptides such as cAM373 and cPD1 are quite active in submicromolar concentrations shows that this structural requirement is only relatively important. Indeed, by removing this structural restraint, the formyl peptide receptor may interact with an unlimited number of peptide fragments from both infectious and host origins to modulate the inflammatory response. The fact that the sex pheromones are found in even higher concentrations in culture filtrates than fMet-Leu-Phe raises the strong possibility that these are physiologically relevant chemotactic agents. Thus the versatility of this receptor to be multifunctional and to give various responses to a large array of peptide stimulants is quite remarkable (35).

Note Added in Proof. While this paper was in review, Ember and Hugli (36) reported on the activity of these *Enterococcus faecalis* sex pheromones for human neutrophils.

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