Vol. 80, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1978 Pages 464-471

ANTIBIOTICS AND PEPTIDES WITH AGONIST AND ANTAGONIST CHEMOTACTIC ACTIVITY

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Received December 2,1977

ABSTRACT

It has been found that the polypeptide antibiotics gramicidin S, tyrocidin and bacitracin, containing Leu-Phe or Ile-Phe sequences, are chemoattractants for neutrophils. Related synthetic pentapeptides containing the sequence Leu-Phe have stronger biological activities, provided the N-terminal residue is acylated. The formylated peptide f-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe is a potent agonist (4×10^{-9}M) whereas the t-butyloxycarbonyl analog is a good antagonist (8×10^{-7}M).

INTRODUCTION

N-formylation of peptides with the sequence L-Met-X-(X = amino acid residue with an aromatic or other hydrophobic side chains) gave products with chemotactic activity for leukocytes (1). A detailed study (2) of structure activity relationships resulted in the synthesis of chemoattractants of high potency (10^{-11}M). In subsequent investigations it was found that there is not an absolute requirement for methionine (3). Methionine may be replaced by norleucine with little loss in chemotactic activity (3).

Since partially characterized peptides of bacterial origin were found to be leukoattractants (4) and the synthetic potent chemotactic peptides contain a Leu-Phe sequence (2,3), it was of interest to determine whether fully defined bacterial products such as gramicidin S, tyrocidin

0006-291X/78/0802-0464$01.00/0

and bacitracin which also have the sequence Leu-Phe or Ile-Phe were attractants. The related peptide L-Phe-D-Leu-L-Phe-D-Leu-L-Phe-OH (Illustration a, R = H) and its N-formylated, N-acetylated, and N-tert-butyloxycarbonylated derivatives (a; R = HCO -, H_3C CO-, $(CH_3)_3C$ O CO-) were also examined for their chemotactic potential.

a R-L-Phe-D-Leu-L-Phe-D-Leu-L-PheOH

We report here the finding that certain polypeptide antibiotics are indeed leukoattractants and interact with the same cell receptor previously demonstrated for synthetic peptides (5).

MATERIALS AND METHODS

Formyl-L-norleucyl-L-leucyl-L-p-[^{3}H]-phenylalanine was prepared from formyl-L-norleucyl-L-leucyl-L-p-chloro-phenylalanine employing a catalytic replacement technique (3).

Gramicidin S, TPCK* and TLCK** were purchased from Calbiochem, bacitracin from Sigma, tyrocidin from Serva Chemicals, New York, New York.

t-Boc-L-phenylalanyl-D-leucyl-L-phenylalanyl-D-leucyl-L-phenylanine [a, R = $(H_3C)_3C$-OCO-] was synthesized by an active ester method beginning with Boc-L-phenylalanine (I). The N-hydroxysuccinimide ester was prepared by allowing I to react with N-hydroxysuccinimide and dicyclohexylcarbodiimide in ethyl acetate (1 h, 0°C; 1 h, 25°). After filtration of the urea and the evaporation of ethyl acetate the product was dissolved in tetrahydrofuran and added to an equal volume of a water suspension of the next amino acid to be coupled and 2 equivalents of $NaHCO_3$. After 1 h of reaction at room temperature the tetrahydrofuran was evaporated, the solution was acidified to pH 2 by the addition of hydrochloric acid and extracted with ethyl acetate. The theoretical weight gain occurred at each step and amino acid analysis indicated quantitative coupling. The final product showed one major spot and minor contaminants in thin layer chromatography (TLC:*** benzene:acetic acid = 7:1). Crystallization from ethyl acetate/petroleum ether gave a material pure by amino acid analysis and TLC in the solvent system given above. Both the formylated and the acetylated pentapeptides were prepared by an analogous procedure utilizing the respective esters of N-hydroxysuccinimide.

In order to obtain the free pentapeptide, the Boc-derivitive (80 mg) was allowed to stand in 10 ml of trifluoroacetic acid at room temperature for 60 min. Following the removal of trifluoroacetic acid under vacuum, the salt was dissolved in 30% ethanol-water. Upon addition of ammonium hydroxide to neutrality the free peptide precipitated. After adjustment

*L-(1-Tosylamido-2-phenyl)ethylchloromethyl ketone
** N-α-p-Tosyl-L-lysylchloromethyl ketone
*** thin-layer chromatography

of its pH to 2.0 with hydrochloric acid, the free peptide was extracted with ethyl acetate. The peptide obtained after solvent evaporation showed the correct amino acid analysis and weight recovery of amino acid. Activated rabbit neutrophils were obtained 12 to 14 hours after the intraperitoneal injection of 0.1% glycogen in phosphate buffered saline (6).

Chemotactic activities were measured using a Boyden chamber (7) by counting the cells which had migrated through and adhered to the underside of a micropore filter which separated two chambers, the upper one containing the cells, the lower, the attractant. In the standard binding assay (5) neutrophils (4.4×10^6 cells) briefly treated with 0.1 mM TPCK, were incubated at 0°C for 1 h in 2 ml of Gey's solution (8) containing 50,000 cpm of [^{3}H]fNorleu-Leu-Phe and the ligand bound to the cells retained after filtration on the glass fiber filter and subsequent washing was counted in a liquid scintillation counter with an efficiency of 40%.

RESULTS

Chemotactic properties of antibiotics and related synthetic peptides

The polypeptide antibiotics are chemotactically active within a range of 10^{-6} - 10^{-5} M (Table 1). The ability of these antibiotics to displace the specific binding of a labelled attractant to neutrophils is closely related to their chemotactic potencies (Table 1) indicating that they modulate chemotaxis through the same receptor as do fMet and fNorleu peptides (5). The formylated pentapeptide is a potent agonist; the potency of the acetylated derivative is lower by approximately two orders of magnitude, and nonacylated peptide shows the lowest activity (Table 1), a result in accord with previous findings on the specificity of acylation (1). The Boc-protected peptide is an inhibitor of chemotaxis; the concentration to effect 50% inhibition (ID_{50}) of chemotactic response to an agonist is 8×10^{-7} M (Table I). A typical dose-response curve is given in Fig. 1 and the calculated ED_{50}s are shown in Table I. The effect of the free pentapeptide and its N-protected derivatives on the specific binding of the labeled attractant [^{3}H] fNorleu-Leu-Phe to the leukocyte is shown in Fig. 2. The curves are essentially parallel indicating that the peptides interact at the same receptor site as the labeled ligand. The calculated ID_{50} values for these compounds are in agreement with their behavioral activities (Table 1).

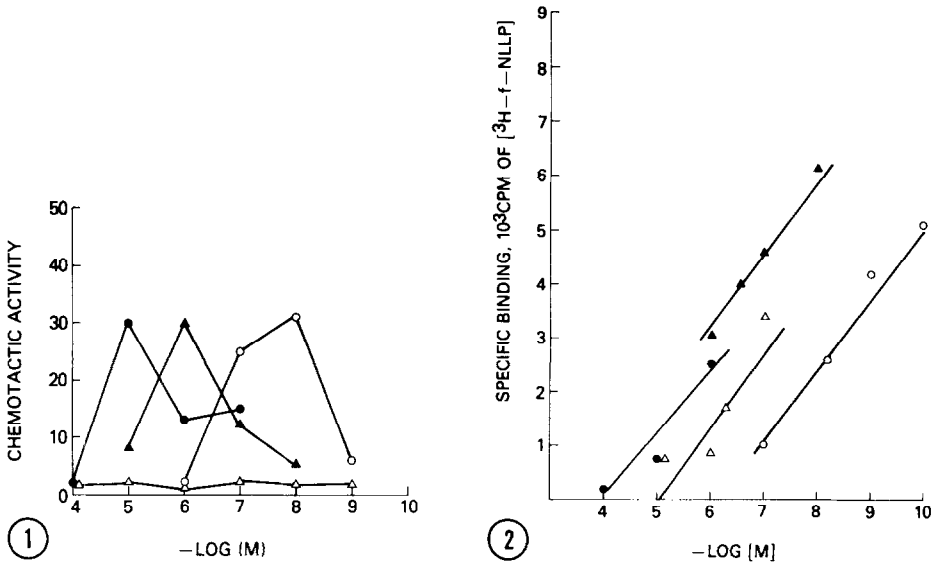

Figure 1. Chemotactic activity of neutrophils in response to H-L-Phe-D-Leu-L-Phe-D-Leu-L-PheOH (•) HCO-L-Phe-D-Leu-L-Phe-D-Leu-L-PheOH (o), H_3CCO-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe-OH (▲) and $(H_3C)_3COCO$-L-Phe-D-Leu-L-PheOH (Δ). The results are expressed as the average number of neutrophils in 10 fields at a magnification of X970 for triplicate samples. The SEM did not exceed 10% for values above 10.

Figure 2. Inhibition of specific binding of (^{3}H)f-Norleu-Leu-Phe to neutrophils by H-L-Phe-D-Leu-L-Phe-D-Leu-L-PheOH (•), HCO-L-Phe-D-Leu-L-Phe-D-Leu-L-PheOH (o), H_3CCO-L-Phe-D-Leu-L-Phe-D-L-Leu-PheOH (▲) and $(H_3C)_3$ COCO-L-Phe-D-Leu-L-Phe-D-Leu-L-PheOH (Δ). ID_{50} for binding was estimated from these plots of specific binding versus concentration of peptides. The values are means of triplicate samples varying less than 10%.

Effect of proteolytic inhibitors

From results with proteolytic inhibitors specific proteases have been invoked as participants in the chemotactic response (9) to peptide attractants. When the effects on neutrophils of the chemotactically active pentapeptide and the antibiotics were tested in the presence of protease inhibitors, similar observations were made (Table II). TPCK, an inhibitor of chymotrypsin, was more effective in inhibiting chemotaxis than TLCK, an inhibitor of trypsin, suggesting a role in chemotaxis for a peptidase with specificity for aromatic residues.

DISCUSSION

The results of the present study show that certain polypeptide

Table 1

Chemotactic Activities of Synthetic Peptides and Peptide Antibiotics

Peptide	Chemotaxis $ED_{50}(M)$[a]	Specific Binding[b] $ID_{50}(M)$	Inhibition of Chemotaxis $ID_{50}(M)$[c]
HCO-L-Met-L-Leu-L-PheOH	7×10^{-11}	3.3×10^{-10}	–
H-L-Phe-D-Leu-L-Phe D-Leu-L-PheOH	4×10^{-6}	1×10^{-5}	-
H_3CCO-L-Phe-D-Leu L-Phe-D-Leu-L-PheOH	5×10^{-7}	4×10^{-7}	-
HCO-L-Phe-D-Leu-L Phe-D-Leu-L-PheOH	4×10^{-9}	2×10^{-8}	-
t-Boc-L-Phe-D-Leu- L-Phe-D-Leu-L-PheOH	-	7×10^{-7}	8×10^{-7}
Gramicidin S	3×10^{-6}	5×10^{-5}	-
Tyrocidin	4×10^{-6}	2×10^{-5}	-
Bacitracin	2×10^{-5}	9×10^{-5}	-

Standard conditions were employed as described in Methods for chemotaxis and binding assays. The values are means of triplicate samples varying less than 10%.

[a] ED_{50} is defined as that concentration of attractant giving a half-maximal chemotactic response.

[b] ID_{50} is defined as that concentration of peptide causing 50% inhibition of specific binding of [^{3}H]-fNorLeu-Leu-Phe on neutrophils.

[c] ID_{50} is defined as that concentration of peptide, not itself an attractant causing 50% inhibition of leucotactic response to the standard potent attractant fMet-Leu-PheOH.

antibiotics are chemotactic for leukocytes and interact with the same receptor on these cells as do synthetic peptides and a crude bacterial factor (5). In efforts to identify active groupings which may contribute to the chemotactic activities of these antibiotics, some synthetic peptides having sequences of Leu-Phe were selected since the most potent defined peptide attractants as well as the antibiotics contained

Table II

Effect of Specific Protease Inhibitors on the Chemotactic Response to Peptides

Peptide	Addition to cells	Percent Inhibition of Chemotaxis[a]
Bacitracin, 0.1mM	TPCK, 0.1mM	100
Bacitracin, 0.1mM	TLCK, 0.1mM	49
Gramicidin S 0.01mM	TPCK, 0.1mM	100
Gramicidin S, 0.01mM	TLCK, 0.1mM	62
Tyrocidin, 0.01mM	TPCK, 0.1mM	100
Tyrocidin, 0.01mM	TLCK,0.1mM	0
HCO-L-Phe-D-Leu-L-Phe-D-Leu-L-PheOH 10nM	TPCK, 0.1mM	84
HCO-L-Phe-D-Leu-L-Phe-D-Leu-L-PheOH, 10nM	TLCK, 0.1mM	0
HCO-L-Phe-D-Leu-L-Phe-D-Leu-L PheOH, 10nM	BTEE[b] 0.1mM	100
HCO-L-Phe-D-Leu-L-Phe-D-Leu-L-PheOH, 10nM	TAME[c] 0.1mM	0

[a] Assays for chemotaxis were carried out as described in Methods.

[b] BTEE, Benzoyl-L-tyrosine ethyl ester

[c] TAME, Tosyl-L-arginine methyl ester

such a sequence. These studies also confirm the great specificity for N-formylation of certain peptides for chemotaxis (1). One of the significant observations here is the finding that replacing the formyl group with t-Boc results in the production of potent inhibitors of chemotaxis (8×10^{-7}M). The formylated pentapeptide, on the other hand, was almost as

potent an agonist as the known most active compound [^{f}-Met-Leu-Phe (2)]. The pentapeptide tested here consisted of alternating D and L residues. Based upon our recent results it seems reasonable to postulate that the all L isomer would be even more active since f-L-Met-L-Leu-L-Phe is more active than f-L-Met-D-Leu-L-Phe and since f-L-Phe-L-Met shows a greater biological effect than its D enantiomer (submitted for publication). Furthermore, other tripeptides containing only L amino acid residues with the -Leu-Phe sequence such as f-Phe-Leu-Phe and f- Nle-Leu-Phe are chemotactic. But the presence of a Leu-Phe sequence may not be the sole requirement for chemotactic activity since polymixin B and EM 49, (gift of Squibb) a new antibiotic similar to polymixin B, do not affect chemotaxis. However, in the case of the chemotactically active antibiotics, their mechanism of action appears to be similar to that proposed for other synthetic peptides (9). That is, the cell response to them is inhibited by the chymotryptic inhibitor, TPCK, more effectively than by the tryptic inhibitor, TLCK, implying the participation of a peptidase specific for aromatic residues in this process.

A polypeptide antibiotic such as gramicidin has been shown to affect cells as an ionophore (10) and more recently to inhibit RNA polymerase (11). What we are reporting here is a new property of these bacterial products (gramicidin, bacitracin, and tyrocidin)—their chemotactic activity for leukocytes. This effect is probably not the result of their ionophoric action since by itself valinomycin (0.1 mM), a known ionophore not having Leu-Phe sequences, affected neither chemotaxis nor binding of the labeled attractant to the cells (unpublished results). Nor does it seem likely that these antibiotics stimulate cell movement by inhibiting RNA synthesis since we have also found (unpublished results) that Actinomycin D does not affect chemotaxis. Rather, the evidence presented here suggests that these polypeptides act through the receptor and peptidase previously implicated in leukotaxis

(5,9). Since leukocyte migration contributes in large part to inflammatory conditions, it may be prudent to take into account the leukotactic properties of these antibiotics in efforts to modulate inflammation through their use.

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