SYNTHESIS AND BINDING CHARACTERISTICS OF AN INTRINSICALLY RADIOLABELED CHEMOTACTIC ACYL TRIPEPTIDE

N^{α}-Formyl–norleucyl–leucyl–phenylalanine

Alan R. DAY, Jeffrey A. RADDING and Richard J. FREER
Department of Pharmacology, Medical College of Virginia, Richmond, Virginia 23298

and

Henry J. SHOWELL and Elmer L. BECKER
Department of Pathology, University of Connecticut Health Center, Farmington, Connecticut 06032

and

Elliot SCHIFFMANN, Barbara CORCORAN and Shrivinivesabhatt ASWANIKUMAR
Laboratory of Development Biology and Anomalies, National Institutes of Health, Bethesda, Maryland 20014

and

Candace PERT
Section on Biochemistry, Adult Psychiatry Branch, National Institute of Mental Health, Bethesda, Maryland 20014, USA

Received 11 March 1977

1. Introduction

A large number of structurally unrelated compounds are known to be chemotactic to neutrophils [1]. The heterogeneity of these molecules as well as the lack of structural information on many of them has prevented any definitive statements as to the nature of the cellular events controlling the chemotactic response. Recent studies in our laboratories have shown that a series of synthetic N^{α}-formyl di-, tri- and tetrapeptides possess specific structural features which are translatable in terms of a specific receptor site on the cell surface of the neutrophil [2].

Identical structure–activity relationships were found for peptide mediated release of β-glucuronidase, lysozyme and chemotactic response. The most potent of these synthetic chemoattractants was N^{α}-formyl. Met.Leu.Phe.OH which had ED_{50}-values of 7×10^{-11} M for chemotaxis and $2.4–2.6 \times 10^{-10}$ M for lysosomal enzyme release [2]. To facilitate biochemical investigation of the mechanisms of chemotaxis and lysosomal enzyme release an intrinsically radiolabeled acyl tripeptide of high specific activity is required. An elegant synthetic pathway towards this end is catalytic dehalotritiation of an halogenated precursor peptide.

Unfortunately in the case of F.Met.Leu.Phe.OH, the sulphur containing side-chain does pose problems not the least of which are catalyst poisoning [3] and desulfuration [4]. To circumvent a similar problem in the synthesis of a high specific activity radiolabeled enkephalin, isosteric replacement of methionine by norleucine proved successful [5]. The isosteric acyl tripeptide F.Nle.Leu.Phe.OH was synthesized and found to possess good activity (ED_{50} 6.6×10^{-10} M chemotaxis and $1.5–1.9 \times 10^{-9}$ M lysosomal enzyme release). Therefore, we synthesized the chlorinated

analog F.Nle.Leu.Phe(Cl).OH and exchanged the ring chlorine atom with tritium (New England Nuclear customer service) to give N^{α}-formyl.Nle.Leu.[*p*-tritio]-Phe.OH. A specific binding assay has been developed using this radiolabeled peptide with rabbit neutrophils. Full details of this procedure are given in ref. [6].

2. Materials and methods

2.1. *Analytical methods*

Thin layer chromatography was performed on pre-coated Merck silica gel 60 F254 glass plates using the solvent system:

(A) *n*-Butanol/water/acetic acid (6:1:1)

(B) Chloroform/methanol/water/acetic acid (60:30:1:4)

(C) Benzene/water/acetic acid (9:1:9).

Peptides were visualized by spraying the plates with ninhydrin and chlorine/*o*-tolidine reagents.

Electrophoreses at pH 2 and pH 5 were carried out routinely on Whatman 3MM paper at 1000 V for 60 min (Isco 490 power pack). Rotary evaporations were carried out in vacuo at a bath temperature of 40°C unless otherwise stated.

2.2. *Chemistry*

Racemic *p*-chlorophenylalanine was trifluoroacetylated [7] and incubated at 37°C with carboxypeptidase A [8]. Optically pure L-*p*-chlorophenylalanine was obtained in good yield (71%); d.p. 248–250°C [ref. [9] 241–243°C]. A single spot was found by thin-layer chromatography R_F^A 0.41; R_F^B 0.28; R_F^C 0.38. $[\alpha]_D^{25} = 23°$ (C 0.4, H_2O) [ref. [9,10] −27.8° (C 0.4, H_2O) and −23° (C 0.5, H_2O)]. Microanalyses for C, H and N gave the expected results. The halogenated amino acid eluted at 133 min under standard 3 h run conditions on a Beckman 119C.

2.3. *Amino acid analyzer*

The free amino acid was protected by reaction with *t*-butyloxycarbonyl (*t*-Boc) azide [11]. A portion of the *t*-Boc–L-*p*-chlorophenylalanine was converted to its cesium salt and coupled to chloromethylated Bio-Beads S-X-1 resin using the method of Gisin [12]. Peptides were synthesized by the Merrifield solid-phase method and were removed from the resin support by reaction with anhydrous hydrogen fluoride in the presence of anisole [13]. The free tripeptide H.Nle.Leu. Phe(Cl).OH was purified by 100 transfers in a Craig-Post countercurrent distribution apparatus using the solvent system *n*-butanol/acetic acid/water, 4:1:5. The peptide was located by quantitative ninhydrin reaction and the contents of tubes 65–80 were pooled, evaporated in vacuo and the residue dissolved in distilled water and lyophilized. The peptide migrated as a single band on electrophoresis at pH 2 and pH 5 (ninhydrin; *o*-tolidine/chlorine visualization) and was homogeneous on thin-layer chromatography R_F^A 0.63; R_F^B 0.39; R_F^C 0.35. Formylation was performed using the method of Sheehan and Yang [14]. The reaction mixture was diluted with 10 vol. water and lyophilized. Thin-layer chromatography indicated that the formylation was quantitative R_F^A 0.79; R_F^B 0.74; R_F^C 0.64. The product was ninhydrin negative, *o*-tolidine positive and judged homogeneous. Amino acid analysis Nle 1.0, Leu 1.07, Phe(Cl) 1.04.

2.4. *Dehalotritiation and chromatography*

Dehalotritiation was carried out by New England Nuclear as a customer service. The radiolabeled compound (14 Ci/mmol) was examined by thin-layer chromatography in solvent systems A, B and C using silica-gel plates. The plates were scanned using a Packard Radiochromatograph model 7201 scanner. Only one peak of radioactivity, which corresponded to the R_F of cold marker peptide, could be detected.

2.5. *Biological assays*

Chemotaxis and lysosomal enzyme-releasing activity were measured as described previously [2].

3. Results and discussion

Previous studies have suggested the presence of a specific high affinity binding site (receptor ?) related to the chemotactic response of neutrophils [2]. The present study was undertaken to allow for more direct investigation of this site via synthesis of a high specific activity radiolabeled ligand. Application of this radiolabeled compound to the study of the chemotactic peptide receptor is described fully in ref. [6]. The ID_{50} given in table 1 is that concentration of cold peptide required to inhibit 50% of the binding of the

Table 1
ED_{50} Concentrations (M)[a]

Compound	Chemotaxis[b]	Lysosomal enzyme release[c]	
		Lysozyme	β-Glucuronidase
F–Met–Leu–Phe[d]	$7.0 \pm 1.7 \times 10^{-11}$	$2.4 \pm 0.31 \times 10^{-10}$	$2.6 \pm 0.32 \times 10^{-10}$
F–Nle–Leu–Phe	$6.6 \pm 1.2 \times 10^{-10}$	$1.5 \pm 0.2 \times 10^{-9}$	$1.9 \pm 0.2 \times 10^{-9}$
F–Nle–Leu–Phe(Cl)	$2.8 \pm 0.6 \times 10^{-9}$	$8.5 \pm 1.1 \times 10^{-9}$	$8.8 \pm 1.5 \times 10^{-9}$

[a] ED_{50} = Concentration of peptide to give 50% maximum chemotactic response or release – data are the average of at least 4–5 individual experiments
[b] Chemotaxis was measured in a Boyden Chember as described previously [2]
[c] Lysosomal enzyme release was measured using cytocholasin B treated cells as described previously [2]
[d] Data taken from ref. [2]

radioactive tracer F.Nle.Leu.Phe.OH. The synthetic route chosen precluded use of the most active peptide in the series because of the presence of a methionine residue. However, we were able to demonstrate that isosteric replacement of the methionine with norleucine produced a peptide with excellent biological activity (table 1). The lack of the sulfur atom also made this peptide highly suitable for intrinsic labeling by catalytic tritiation. Therefore, the *p*-chlorophenylalanine analog was prepared by us and dehalotritiated as a customer service by New England Nuclear. The resulting product was highly labeled (14 Ci/mmol) and found to be homogeneous by thin-layer chromatography in solvent systems A, B and C (fig.1). The radioactive peak corresponded to cold marker peptide in each system. Figure 2 demonstrates the close correlation between specific binding, chemotaxis and lysosomal enzyme activities of six analogs (plotted as the ED_{50}). The linear regression equation relating specific binding (X) to chemotaxis (Y) is

$$Y = (0.96)\,X - 0.86.$$

For specific binding (X) and lysozyme activity (Y)

$$Y = (0.95)\,X - 0.48.$$

Finally the equation relating specific binding (X) to β-glucuronidase activity (Y) is

$$Y = (0.97)\,X - 0.27.$$

The correlation coefficients in each case are > 0.99. The slope of each line is close to 1.0 indicating that each of the biological properties of the peptides is proportional linearly to 50% occupancy of available binding sites as determined in the specific binding assay. The lower concentration of peptide required

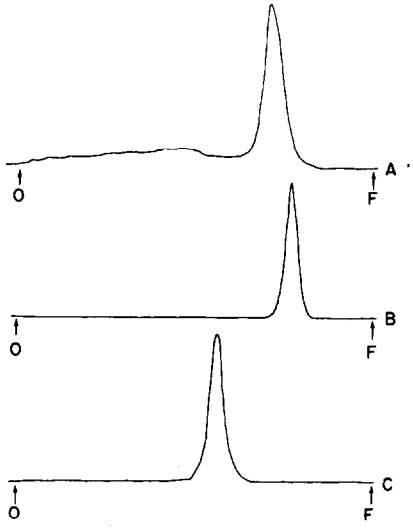

Fig.1. Radiochromatogram scans of *N*-formyl–norleucine–leucyl–[*p*-tritio]phenylalanine following thin-layer chromatography carried out in systems A, B and C as described in Materials and methods. Scanning rate was 2.0 cm/min. Linear ranges were 3×10^4 cpm (A) and 1×10^5 cpm (B and C) and time constants were 10 (A) and 3 (B and C). Radiolabeled load gave 60–90% of the full scale deflection in the linear range indicated. (O) Origin, (F) solvent front.

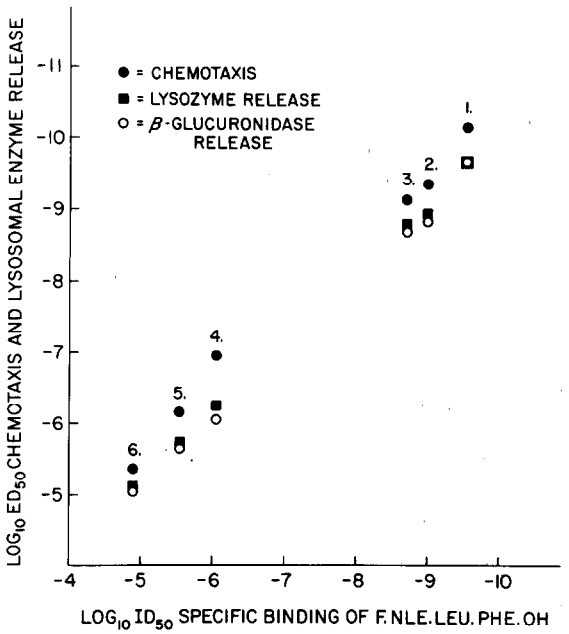

Fig.2. Correlation diagram for specific binding versus chemotaxis (●), lysozyme release (■) and β-glucuronidase release (○). Data plotted as ED_{50} for 6 *N*-formyl tripeptides. (1) F–Met–Leu–Phe.OH, (2) F–Ethioninyl–Leu–Phe.OH, (3) F–Norleucyl–Leu–Phe.OH, (4) F–Phe–Leu–Phe.OH, (5) F–Met–Leu–Arg.OH and (6) F–Gly–Leu–Phe.OH.

to induce chemotaxis is of particular interest when one considers that the cells are responding to a concentration gradient.

It is interesting from a structure–activity point of view that the sulfur of the methionine side-chain is not absolutely essential for biological activity (table 1). It does, however, enhance activity as indicated by the fact that the norleucyl analog had only 10.6% the activity of the parent F.Met.Leu.Phe.OH in the chemotactic assay. Additional studies have been carried out to determine the structure–activity relationships in this position. Also worthy of note is the 4–6-fold decrease in activity of the halogenated peptide compared to the parent norleucine peptide. This latter observation might suggest that the area of the receptor interacting with the aromatic ring is spatially restricted. It will be interesting to see if this area of the receptor can be further probed by other modifications of the phenylalanyl residue.

Acknowledgements

This work was supported by NIDR Contract DE-52477.

References

[1] Wilkinson, P. C. (1974) in: Chemotaxis and Inflammation, Churchill Livingston, p.54, Edinburgh, London.
[2] Showell, H. J., Freer, R. J., Zigmond, S. H., Schiffmann, E., Aswanikumar, S., Corcoran, B. and Becker, E. L. (1976) J. Exp. Med. 143, 1154–1169.
[3] Schröder, E. and Lübke, K. (1965) in: The Peptides, Vol. 1, p. 241, Academic Press, New York and London.
[4] Guttmann, S. and Boissonnas, R. A. (1958) Helv. Chim. Acta. 41, 1852–1867.
[5] Day, A. R. and Freer, R. J. (1977) submitted for publication.
[6] Aswanikumar, S., Corcoran, B., Schiffmann, E., Day, A. R., Freer, R. J., Showell, H. J., Becker, E. L. and Pert, C. B. (1977) Biochem. Biophys. Res. Commun. 74, 810–817.
[7] Weygand, F. and Geiger, R. (1956) Chem. Ber. 89, 647–652.
[8] Vine, W. H., Brueckner, D. A., Needleman, P. and Marshall, G. R. (1973) Biochemistry 12, 1630–1637.
[9] Houghten, R. A. and Rapoport, H. (1974) J. Med. Chem. 17, 556–558.
[10] Kovács, J., Teplán, I. and Mezö, I. (1975) Acta Chim. Acad. Sci. Hung. 84, 109–114.
[11] Anderson, G. W. and McGregor, A. C. (1957) J. Am. Chem. Soc. 79, 6180–6183.
[12] Gisin, B. F. (1973) Helv. Chim. Acta. 56, 1476–1482.
[13] Stewart, J. M. and Young, J. D. (1969) in: Solid Phase Peptide Synthesis, W. H. Freeman, San Francisco.
[14] Sheehan, J. C. and Yang, D. H. (1958) J. Am. Chem. Soc. 80, 1154–1158.