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Polymeric analogues of N-formyl peptides are potent activators of degranulation and superoxide production by human neutrophils

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Summary: Analogues of N-formyl methionyl leucyl phenyalanine possessing three and four peptide units grafted onto an inert carbon skeleton were tested as activators of human polymorphonuclear leukocytes. For the two responses studied, degranulation and respiratory burst, the polymeric analogues showed two maxima of activity, one at the same concentration as the monomer, the other one at a concentration 100- to 1000-fold lower. The potency of the polymers with respect to the monomer is discussed in terms of receptor clustering. The similarity of the dose-response curves for superoxide production and lysozyme secretion indicates that the early transmembrane signalling events are identical for the two responses studied.

N-formyl peptides, which are bacterial metabolites, are known chemoattractants for polymorphonuclear leukocytes (PMN) (1,2) which sense their presence via specific receptors (3,4). Ligand binding not only induces chemotactic responses but also cell aggregation and adhesion, phagocytosis, release of lysosomal enzymes and production of superoxide anion (5-7). Chemotaxis usually requires 10- to 100-fold lower concentrations of chemotactic peptides than secretory functions (8,9).

Two populations of formyl peptide receptors, with different affinities for the ligand were detected on PMN membranes in equilibrium-binding studies (10,11). The equilibrium between these populations can be affected by diverse pharmacological agents and by guanine nucleotides (5-7,12-16). In addition, a third desensitized state arising after ligand binding has been described (6,7,15-9). Numerous studies showed that the kinetics and intensity of responses depend on level of receptor occupancy, rate and duration of ligand binding (7,15,16,20,21). Because of the many pharmacological tools available to manipulate the system and of the variety of responses elicited by ligand binding to the formyl peptide receptors, PMN constitute an excellent model for the study of signal transduction.

Abbreviations used: fMLP : N-formyl-methionyl-leucyl phenylalanine; fMLP-OCH_3 : N-formyl-methionyl-leucyl-phenylalanine methyl ester; HBSS : Hank's balanced salt solution; PMN : polymorphonuclear leukocytes.

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Formyl-methionyl-leucyl-phenylalanine (fMLP) is the most extensively used of formyl peptides. Nevertheless, many synthetic analogues have been tested and found to act as agonists or antagonists (1,2,8,22-28). Among the agonists synthesized are a series of tetramers of fMLP amides bridged to tetraazacycloalkanes through 6 carbon spacers (28). These compounds were found to elicit lysozyme secretion from human neutrophils at concentrations 500- to 1500- fold lower than that of the model compound formyl-methionyl-leucyl-phenylalanine methyl ester (fMLP-OCH_3). This enhanced activity was ascribed to a cooperative response of the membrane receptors. In this paper we report further results obtained with two of these tetrameric clusters, as well as with a new trimeric cluster; we compared their potency for eliciting lysozyme secretion and superoxide anion production to that of fMLP-OCH_3.

Materials and Methods

Materials. The structure of the compounds used is shown in Figure 1. The synthesis of compounds **1-3** is described in (28), that of compound **4** in (29). The chemoattractants were dissolved in dimethylsulfoxide. The organic solvent concentration in the assays as well as in the controls was 1%.

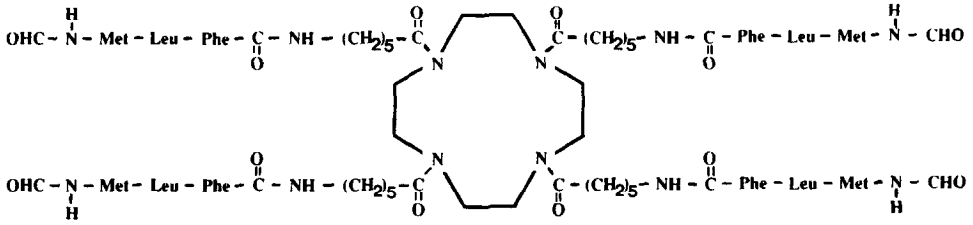
Isolation of human neutrophils. PMN were harvested from peripheral venous blood of human volunteers according to the technique of Böyum (30) with a few modifications. After a 1-h dextran sedimentation, the leukocyte-rich plasma was layered over Ficoll-Hypaque and centrifuged for 30 min at 400xg. Residual red blood cells were removed from the resultant PMN pellet by a 5-min hypotonic lysis. The preparation usually contained 97% PMN and 3% lymphocytes. Cell viability was tested with the trypan blue exclusion test. PMN were resuspended in calcium and magnesium free Hank's balanced salt solution (HBSS) (pH = 7.5) at 10^8 cells/ml for superoxide assays or at 5.10^8 cells/ml for lysozyme determinations and kept at 4°C until use.

Assay of superoxide ion production. The formation of superoxide was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* in a continuous spectrophotometric assay (30). Duplicate reaction mixtures containing PMN (10^6 cells/ml), cytochrome *c* (200 µM), cytochalasin B (5 µg/ml) and Ca^{2+}- and Mg^{2+}-containing HBSS were allowed to equilibrate at 37°C for 2 min in spectrophotometer cells. The reference, in addition, contained superoxide dismutase (10 µg/ml). The reaction was initiated simultaneously in the two cuvettes by addition of the required amount of stimulant. The reaction was continuously monitored at 550 nm for 2 min with a Uvikon 930 spectrophotometer. The maximal initial rate of cytochrome *c* reduction was calculated by using $\varepsilon^{red\_ox} = 21\ mM^{-1}\ cm^{-1}$.

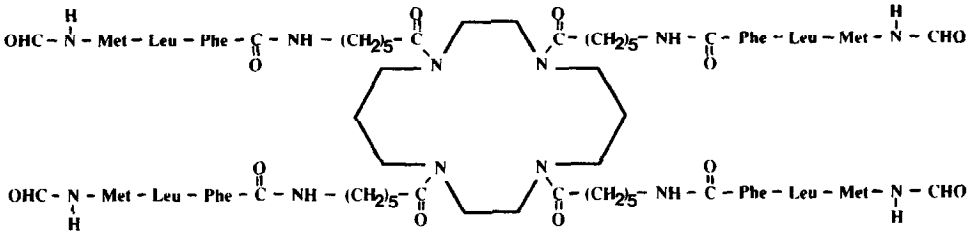
Assay of lysozyme release. The reaction mixtures contained cells (5.10^6/ml), cytochalasin B (5 µg/ml) and bovine serum albumin (2 mg/ml) in a total volume of 1ml in Ca^{2+}- and Mg^{2+}-containing HBSS. They were equilibrated for 5 min at 37°C before addition of the activator (5 µl). Five min. later the cells were removed by centrifugation at 1500xg for 5 min at 4°C (8). Aliquots of the supernatants were used for determination of lysozyme activity as described (31).

Results

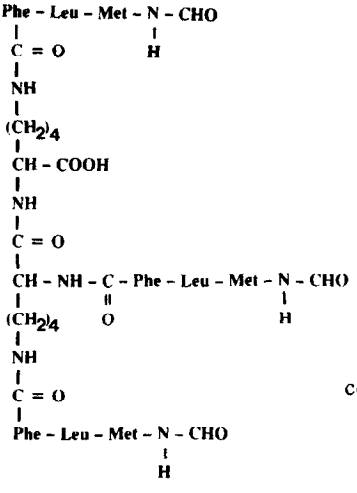
Fig 2A illustrates the classical dose-response curve for superoxide anion production obtained with monomeric compound **1**; on the other hand, the dose-response curve obtained with the trimeric cluster **4** shown in Fig.2b, presents two

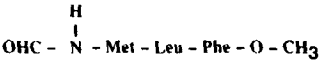


compound 2



compound 3





compound 1

compound 4

Fig.1. Structure of the fMLP analogues used in this study.

maxima of activity, one at low concentrations and another one in roughly the same concentration range as the monomer. The tetrameric clusters gave analogous bimodal curves. Similar aspects were obtained for dose-response curves for lysozyme secretion. For experiments such as those of Fig 2b, each maximum was considered independently for determination of an EC_{50}. The values of EC_{50}^1 and EC_{50}^2 for superoxide production are reported in Table 1, those for lysozyme secretion in Table 2. The maximal rates of superoxide production were similar for the four compounds tested, and the rate at the second maximum was on the average 55 to 62% of that at the first maximum, whatever the polymeric analogue.

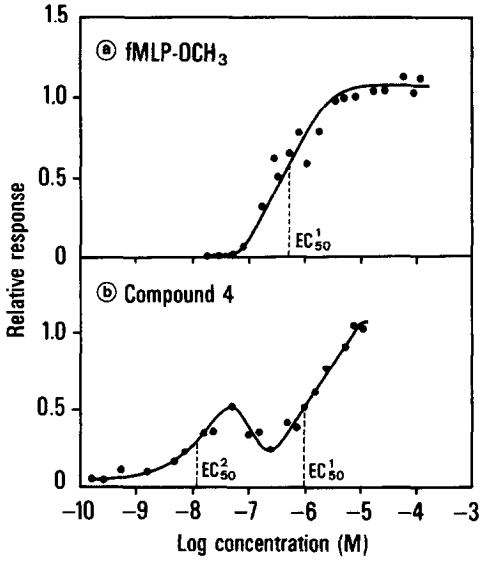


Fig. 2. Typical dose-response curves for superoxide anion production elicited by the monomer **1** and a polymer (trimer **4**).
The results of each independent experiment carried out with a different blood sample were normalized by taking the response in the analogue concentrations range 1 to 5 - 10^{-6} M as equal to 1. The normalized experimental points from several experiments were then combined to give plot a (monomer **1**, 8 independent experiments) and plot b (trimer **4**, 4 independent experiments). The response was taken to be the maximal rate of superoxide production at each analogue concentration.

Discussion

The salient feature of the present results is the existence of two maxima of activity for the polymeric clusters both for superoxide production and lysozyme secretion. EC_{50} values for the first maximum are within experimental error the same as that of the reference monomer. If this first maximum arose from the presence of contaminant monomer, it should occur at a higher concentration, since the monomer would only represent a fraction of the material. Furthermore, NMR spectra carried out after these results were obtained confirmed that the material used was identical to the initial

Table 1. EC_{50} values for superoxide anion production

Compound	EC_{50}^1 (M)	EC_{50}^2 (M)
1	$1.1 \pm 0.4 \times 10^{-6}$	
2	$3.0 \pm 0.9 \times 10^{-6}$	$2.2 \pm 1.3 \times 10^{-9}$
3	$3.2 \pm 1.6 \times 10^{-6}$	$2.7 \pm 0.7 \times 10^{-9}$
4	$1.9 \pm 0.7 \times 10^{-6}$	$10.4 \pm 6.5 \times 10^{-9}$

The values presented are the average ± SD of EC_{50}^1 and EC_{50}^2 values determined for each individual dose-response curve (8 experiments for monomer **1** and 4 experiments each for compounds **2-4**).

Table 2. EC_{50} values for lysozyme release

Compound	EC_{50}^1 (M)	EC_{50}^2 (M)
1	$2.3 \pm 0.4 \times 10^{-7}$	
3	$2.30 \pm 0.5 \times 10^{-7}$	$2.9 \times 0.6 \times 10^{-10}$
4	$1.2 \pm 0.4 \times 10^{-7}$	$5.7 \pm 0.3 \times 10^{-10}$

The values presented are the average ± SD of EC_{50}^1 and EC_{50}^2 determined for two individual dose-response curves for each compound. There was not enough material left to determine the effect of compound 2 on lysozyme release.

preparations. EC_{50} values for the second maximum are about 3 orders of magnitude lower than those for the first maximum. This is much larger than the factor of 3 or 4 expected from a simple additive effect. Only this second maximum, at comparable concentrations, was observed in previous work for lysozyme secretion induced by compounds **2** and **3** (28).

In view of the polymeric nature of the analogues, we wish to suggest the following explanation for the unusual aspect of dose-response curves such as that of Fig 2b. The ascending limbs of the curve at low concentrations would represent the response elicited by polymeric binding of the analogues. With further concentration increases, the polymers would start competing with each other for receptors, just as immunoglobulins compete for antigen during immunoprecipitation. Binding of the clusters would progressively become monomeric with further concentration increases, and this would explain the other maximum with the same EC_{50} value as that of the monomer.

If the existence of the low concentration maximum were only due to increased affinity of the polymers relative to the monomer owing to cooperative binding of the fMLP units of each cluster, we would not expect to see the descending limb of the curve. Indeed, as polymers would start competing with each other, the degree of receptor occupancy would keep increasing with increasing concentration, in other words a single smooth curve such as that of the monomer (Fig. 2a), but displaced toward lower concentrations, should be observed. Therefore the characteristics of the bimodal dose-response curves must arise from special features associated with the polymeric nature of the analogues : they could, as suggested previously (28), induce receptor clustering. Receptor aggregation or capping has been studied in diverse systems (32-35) including neutrophils (16,36,37); manipulation of the phenomenon may be a useful approach in drug design (28,38). At the molecular level, it not clear whether the enhancing effects observed with the multivalent fMLP analogues could be related at all to the lateral segregation effects in the membrane described for the fMLP receptor after ligand binding (7,16).

The second interesting point derived from our results is the parallelism of the effects observed for the two responses studied. Several reports appeared in the

literature, describing differential effects of pharmacological agents on fMLP-induced superoxide generation and degranulation : the diacylglycerol kinase inhibitor R 59022 was found to potentiate superoxide production but not secretion (39); protein I, a translocatable ion channel from *Neisseria gonorrhoeae*, as well as a monoclonal antibody against human neutrophils, inhibited exocytosis without affecting the respiratory burst (40,41). Several diacylglycerols also inhibited agonist-induced exocytosis but stimulated superoxide production (42), while staurosporine had no effect on agonist-dependent exocytosis but inhibited the burst (43). While some of these agents clearly act at a late point in the transduction cascade, our results with agonist analogues indicate that degranulation and superoxide anion production must share the transmembrane signalling events proximal to ligand binding to the fMLP receptor.

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