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Differences Arising in Human Neutrophil Activation Passing from *N*-Formyl to *N*-Acetyl-oligopeptides

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N-formyl- and *N*-acetyl-peptides were synthesized and compared in order to understand which features can best elicit biological responses. The behavior of *N*-formyl-peptides confirms the previously found sequential obligations in the residues, while acetyl-derivatives do not seem suitable for an efficacious stimulation of human neutrophils.

Key Words: human neutrophils; formyl-peptides; acetyl-peptides; chemotactic activity; superoxide anion production; lysozyme release.

INTRODUCTION

Regulation of the level of circulating neutrophils is very important because they control microbial infection by removing and killing pathogens. Neutrophils detect invading agents by means of signal molecules called chemoattractants, generated by bacterial infection and tissue damage (*1*); they express specific G-protein coupled receptors (*2*) for chemoattractants, which enable them to sense invading foreign particles and to approach the site of infection by triggering cytoskeletal reorganization and cell shape change (*3*,*4*).

N-formyl-Met-Leu-Phe-OH (fMLP), which derives from bacterial sources or disrupted mitochondria, is the reference chemotactic peptide together with its synthetic methylester derivative for-Met-Leu-Phe-OMe (fMLP-OMe).

Neutrophil chemotaxis toward the site of invasion is activated by low concentrations of the ligand (*5*); in contrast, its high concentration activates an array of other responses, including the release of hydrolytic enzymes from the granules into the extracellular fluid, and the triggering of a "respiratory burst," which is characterized by the generation of superoxide anion (O_2^-) (*6*).

Although it seems well-established that the formyl as amino-terminal blocking group is crucial for both binding and biological activity (*7*), a number of studies have

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nevertheless been performed on acetyl-oligopeptides, but the picture is still unclear. While it seems ascertained that acetyl-Met-Leu-Phe-OMe (Ac-MLP-OMe) is devoid of any activity, biological responses have been found for acetyl-peptides containing a varied and/or longer sequence: e.g., acetyl-peptides containing five or more residues elicit a potent chemotactic activity for human neutrophils (*8,9*).

These observations prompted us to try and give a final answer to the question whether a biological response is present (and if so, which) when the sequence in the prototype peptide is changed, and/or when the formyl group is replaced by an acetyl group in short peptides.

For this purpose, formyl- and acetyl-tri and tetra-peptides were synthetized to test their efficiency in triggering biological responses in human neutrophils. Chemotaxis, superoxide anion production, and lysozyme release of Ac-Met-Leu-Phe-OMe **1**, for-Met-Phe-Leu-OMe **2**, Ac-Met-Phe-Leu-OMe **3**, for-Met-Phe-Leu-Val-OMe **4**, Ac-Met-Phe-Leu-Val-OMe **5**, for-Met-Leu-Phe-Val-OMe **6**, and Ac-Met-Leu-Phe-Val-OMe **7** were compared with those observed for the reference peptide fMLP-OMe.

These sequences have been chosen because they are present in the N-terminal sequence of the calpain small subunit and exhibit chemotactic activity (*8*).

MATERIALS AND METHODS

Chemistry

The ^{1}H-NMR spectra were recorded in deuterated chloroform ($CDCl_3$) and dimethylsulphoxide (DMSO-d6) on a Bruker AC200 spectrometer at 200 MHz. Chemical shifts are expressed as δ (ppm) related to the TMS signal.

Optical rotations were determined in MeOH at 20°C with a Perkin–Elmer Model 241 polarimeter.

Melting points were determined on a Reichert–Kofler block and are uncorrected.

Thin layer chromatography was performed on precoated silica gel F_{254} (Merck) with the solvent system: methylene choloride/toluene/methanol 17/1/2.

Satisfactory microanalyses were obtained for all compounds, analytical results being within $\pm 0.4\%$ of the theoretical values.

Amino acids hydrochlorides as well as their Boc and acetyl derivatives were purchased from Fluka. Removal of the Boc group was performed by treatment with a 1:1 mixture of trifluoroacetic acid (TFA)-$CHCl_3$. Peptide coupling was achieved (i) by the racemization-free mixed-anhydride method with isobutylchloroformate (IBCF) and triethylamine (TEA) (*10*), and (ii) by the 1-hydroxy-benzotriazole (HOBt)–*N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC) method (*11*), while the formyl group was introduced according the *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) method (*12*).

Purification of all the final products were achieved by reverse phase HPLC analysis on a Waters Delta Prep 3000 and revelation with UV spectrometer Waters 484 at 220 nm using, as stationary less polar phase a Delta Pack C 18-300 A column (30 mm × 30 cm, particles 15 μm) with a proper eluting system.

Ac-Met-Leu-Phe-OMe **1**

The peptide was synthesized following standard procedures in solution (Fig. 1). Solid (mp.185–187°C; R_f 0.53; $[\alpha] = -15.5°$, $c = 1$). ^{1}H-NMR ($CDCl_3$): 0.88 and

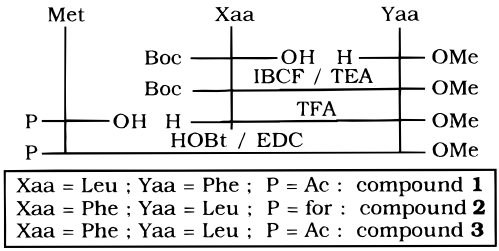

FIG. 1. Scheme of synthesis of compounds **1, 2**, and **3**. All the abbreviations are reported under Materials and Methods.

0.91 (6H; $2CH_3$; 2d; $J = 5.22$ Hz); 1.35–1.60 (2H; CH_2; m); 1.90–2.05 (3H; CH_2+CH; m); 2.00 (3H; CH_3CO; s); 2.08 (3H; SCH_3; s); 2.49 (2H; CH_2; m); 3.06 and 3.14 (2H; CH_2; 2dd; AB of ABX; $J_{AB} = 14.41$ Hz; $J_{AX} = 5.60$ Hz; $J_{BX} = 6.27$ Hz); 3.70 (3H; OCH_3; s); 4.45–4.90 (3H; 3CH; m); 6.72 (1H; NH; d; $J = 8.14$ Hz); 6.93 (1H; NH; d; $J = 7.83$ Hz); 7.07–7.13 (3H; C_6H_5+NH; m); 7.23–7.28 (3H; C_6H_5; m).

For-Met-Phe-Leu-OMe **2**

The peptide was synthesized following standard procedures in solution (Fig. 1). Solid (mp. 176–178°C; R_f 0.49; $[\alpha] = -70.3°$, $c = 1$). ^{1}H-NMR ($CDCl_3$/DMSO-d6 1:1): 0.84 and 0.87 (6H; $2CH_3$; 2d; $J = 7.08$ Hz); 1.45–1.95 (5H; $2CH_2$+CH; m); 1.97 (3H; SCH_3; s); 2.31 (2H; CH_2; t; $J = 7.68$ Hz); 2.80 and 3.05 (2H; CH_2; 2dd; AB of ABX; $J_{AB} = 13.02$ Hz; $J_{AX} = 4.38$ Hz; $J_{BX} = 8.59$ Hz); 3.60 (3H; OCH_3; s); 4.25–4.45 (2H; 2CH; m); 4.55 (1H; CH; m); 6.72 (1H; NH; d; $J = 8.14$ Hz); 7.17–7.13 (5H; C_6H_5; s); 7.97 (1H; HCO; s); 8.02–8.16 (2H; 2NH; m).

Ac-Met-Phe-Leu-OMe **3**

The peptide was synthesized following standard procedures in solution (Fig. 1). Solid (mp.168–170°C; R_f 0.45; $[\alpha] = -42.7°$, $c = 1$). ^{1}H-NMR ($CDCl_3$): 0.87 and 0.90 (6H; $2CH_3$; 2d; $J = 5.62$ Hz); 1.45–1.65 (2H; CH_2; m); 1.80–2.02 (3H; CH_2+CH; m); 1.96 (3H; CH_3CO; s); 2.05 (3H; SCH_3; s); 2.47 (2H; CH_2; m); 3.06 (2H; CH_2; m); 3.69 (3H; OCH_3; s); 4.43–4.89 (3H; 3CH; m); 6.62 (1H; NH; d broad); 6.72 (1H; NH; d broad); 6.95 (1H; NH; d broad); 7.10–7.40 (5H; C_6H_5; m).

For-Met-Phe-Leu-Val-OMe **4**

The peptide was synthesized following standard procedures in solution (Fig. 2). Solid (mp.215–218°C; R_f 0.65; $[\alpha] = -9.1°$, $c = 1$). ^{1}H-NMR ($CDCl_3$/DMSO-d6 1:1): 0.85–0.87 (12H; $4CH_3$; 2d); 1.42–1.53 (2H; CH_2; m); 1.54–1.90 (3H; CH_2+CH; m); 1.96–2.09 (1H; CH; m); 2.03 (3H; SCH_3; s); 2.29–2.36 (2H; CH_2; m); 2.81 and 3.02 (2H; CH_2; 2dd); 3.61 (3H; OCH_3; s); 4.18 (1H; CH; m); 4.35 (1H; CH; m); 4.53 (1H; CH; m); 7.17 (5H; C_6H_5; m); 7.69 (1H; NH; d; $J = 8.27$ Hz); 7.83 (1H; NH; d; $J = 6.99$ Hz); 7.85 (1H; HCO; s); 7.90 (1H; NH; d; J=8.17 Hz); 8.07 (1H; NH; d; $J = 8.31$ Hz).

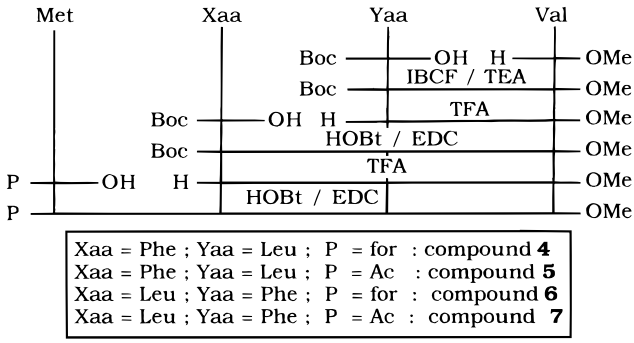

FIG. 2. Scheme of synthesis of compounds **4, 5, 6**, and **7**. All the abbreviations are reported under Materials and Methods.

Ac-Met-Phe-Leu-Val-OMe **5**

The peptide was synthesized following standard procedures in solution (Fig. 2). Solid (mp.>220°C; R_f 0.56; $[\alpha] = -27.1°$, $c = 1$). ^{1}H-NMR (DMSO); 0.89–0.96 (12H; 4CH_3; 2d); 1.47–1.85 (3H; CH_2+CH; m); 1.92 (3H; SCH_3; s); 2.07 (3H; CH_3; s); 2.15 (1H; CH; m); 2.49 (2H; CH_2; t; $J = 7.0$ Hz); 3.12 (2H; CH_2; AB of ABX; $J_{AB} = 6.50$ Hz; $J_{AX} = 3.17$ Hz; $J_{BB} = 2.54$ Hz); 3.74 (3H; OCH_3; s); 4.41–4.58 (3H; 3CH; m); 4.75 (1H; CH; m); 6.31 (1H; NH; d; $J = 6.63$ Hz); 6.95 (1H; NH; d; $J = 10.00$ Hz); 7.02 (1H; NH; d; $J = 9.12$ Hz); 7.10–7.40 (7H; C_6H_5+NH; m).

For-Met-Leu-Phe-Val-OMe **6**

The peptide was synthesized following standard procedures in solution (Fig. 2). Solid (mp.218–220°C; R_f 0.61; $[\alpha] = -48.7°$, $c = 1$). ^{1}H-NMR ($CDCl_3$): 0.85–0.88 (12H; 4CH_3; 2d); 1.42–1.60 (2H; CH_2; m); 1.90–2.20 (5H; 2CH_2+CH; m); 2.04 (3H; SCH_3; s); 2.47 (2H; CH_2; m); 3.05 (2H; CH_2; d; $J = 6.82$ Hz); 3.70 (3H; OCH_3; s); 4.44 (1H; CH; m); 4.71 (1H; CH; m); 4.96 (1H; CH; m); 7.18 (1H; NH; d broad); 7.21 (5H; C_6H_5; m); 7.37 (1H; NH; d broad); 7.57 (1H; NH; d broad); 7.79 (1H;NH; d broad); 8.21 (1H; HCO; s).

Ac-Met-Leu-Phe-Val-OMe **7**

The peptide was synthesized following standard procedures in solution (Fig. 2). Solid (mp.>220°C; R_f 0.69; $[\alpha] = -51.4°$, $c = 1$). ^{1}H-NMR ($CDCl_3$); 0.83–0.89 (12H; 4CH_3; 2d); 1.48 (2H; CH_2; m); 1.93–2.17 (4H; CH_2+2CH; m); 2.03 (3H; SCH_3; s); 2.08 (3H; CH_3; s); 2.49 (2H; CH_2; m); 3.08 (2H; CH_2; m); 3.70 (3H; OCH_3; s); 4.42 (2H; 2CH; m); 4.61–4.80 (2H; 2CH; m); 6.59 (1H; NH; d; $J = 7.03$ Hz); 6.68 (1H; NH; d; $J = 7.14$ Hz); 7.00 (1H; NH; d; $J = 7.78$ Hz); 7.10–7.40 (6H; C_6H_5+NH; m).

Biological Essay (13)

Human neutrophils were purified employing the standard techniques of dextran sedimentation of heparinized blood, followed by centrifugation on Ficoll–Paque and

hypotonic lysis of red cells. The cells were washed twice and resuspended in Krebs–Ringer phosphate containing 0.1% w/v glucose (KRPG), pH 7.4. The percentage of neutrophils was 98–100% pure and ≥99% viable as determined by Tripan blue exclusion test.

Random locomotion was performed with a 48-well microchemotaxis chamber and the migration into the filter was evaluated by the method of leading-front. The actual control random movement is 32 μm ± 3 SE of 10 separate experiments done in duplicate.

Chemotaxis was studied by adding each peptide to the lower compartment of the chemotaxis chamber. Peptides were diluted from a stock solution (10^{-2} M in DMSO) with KRPG containing 1 mg/ml of bovine serum albumin and used at concentrations ranging from 10^{-12} to 10^{-5} M. Data were expressed in terms of chemotactic index (C.I.), which is the ratio: migration toward test attractant minus migration toward the buffer/migration toward the buffer.

Superoxide anion production was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c modified for microplate based assays. The tests were carried out in a final volume of 200 μl containing 4×10^{-5} neutrophils, 100 nmol cytochrome c, and KRPG. At zero time, different amounts (10^{-8}–5×10^{-5} M) of each peptide were added and the plates were incubated into a microplate reader (Ceres 900, Bio-Tek Instruments, Inc.) with the compartment T set at 37°C. Absorbance was recorded at wavelenghts of 550 and 468 nm. Differences in absorbance at the two wavelenghts were used to calculate nanomoles of O_2^- produced, using molar extinction coefficient for cytochrome c of 15.5 mM^{-1} cm^{-1}. Neutrophils were preincubated with 5 μg/ml cytochalasin B for 5 min prior to activation by peptides.

Granule enzyme assay. Release of neutrophil granule enzymes was evaluated by determining lysozyme activity modified for microplate-based assays. Cells were incubated in microplates wells in the presence of each peptide in a final concentration of 10^{-8}–5×10^{-5} M for 15 min at 37°C. The plates were then centrifuged for 5 min at 400*g* and the lysozyme was quantified nephelometrically by the rate of lysis of cell wall suspension of *Micrococcus lysodeikticus*. Neutrophils were preincubated with 5 μg/ml cytochalasin B for 15 min at 37°C prior to activation by peptides. Reaction rate was measured with a microplate reader at 465 nm. Enzyme was expressed as net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 mg/liter $\times10^7$ cells/min.

Statistical analysis. The nonparametric Wilcoxon test was used in the statistical evaluation of differences between groups.

RESULTS

Chemotaxis is shown in Fig. 3. Blocking the N-terminal amino group by an acetyl group in tripeptides **1** and **3** leads to a loss of the chemotactic activity; the same behavior is seen for derivative **2**, which carries the formyl group. A more elaborate pattern emerges from the evaluation of tetrapeptides. Compounds **4, 6**, and **7** show statistically significant activities as chemoattractants. In particular, compound **6** is more efficient and potent than the parent peptide at a concentration of 10^{-11} M. The corresponding acetylated **7** and formylated **4**, where the Phe residue is shifted at position 2, evidence a reduced efficiency (activity peak only at the concentration of

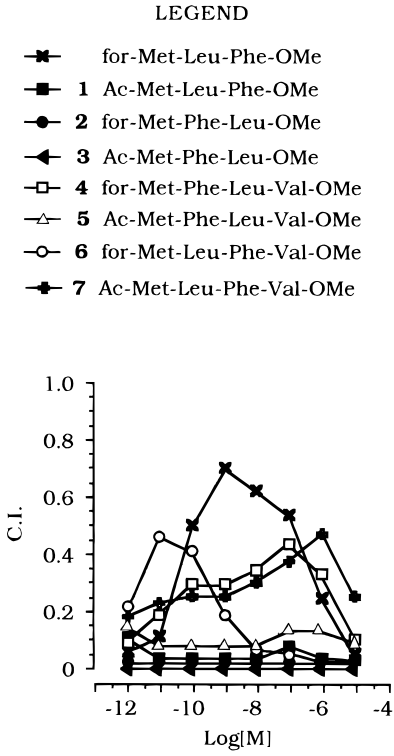

FIG. 3. Chemotactic activity of fMLP-OMe and its analogues toward human neutrophils. The points are the means of five separate experiments done in duplicate. SE are in the 0.02–0.09% chemotactic index range.

10^{-7} M) and less potency (about 35%) than fMLP-OMe. The corresponding acetyl-petide **5**, conversely, is totally devoid of any activity.

Superoxide anion production is shown in Fig. 4. In acetyl-tripeptide derivatives **1** and **3** the response is totally absent, while the acetylated tetrapeptides **5** and **7** show their maximal potency only at 10^{-5} M, thus exhibiting an efficacy ten times less than fMLP-OMe. In particular, the potency of **7** is as high as the control, while compound **5** shows an activity about one-third of the control. All the formylated peptides, however, are able to activate O_2^- production with an order of potency at 10^{-5} M of: fMLP-OMe $>$ **6** $>$ **4** $>$ **2**.

Hydrolytic enzyme release is statistically not significant in acetyl-tripeptides **1** and **3**, while formyl-tripeptide **2** reaches the value of the control only at the concentration of 10^{-5} M. Acetylated tetrapeptides seem to be sensitive to the sequence of the chain residues: in fact, compound **5** reaches its maximum only at a concentration of 10^{-5} M, while compound **7** exhibits a similar pattern of activity to the reference at all the concentrations tested. Formyl-tetrapeptides **4** and **6** possess a more potent degranulating activity than fMLP-OMe at 10^{-6} and 10^{-7} M, respectively.

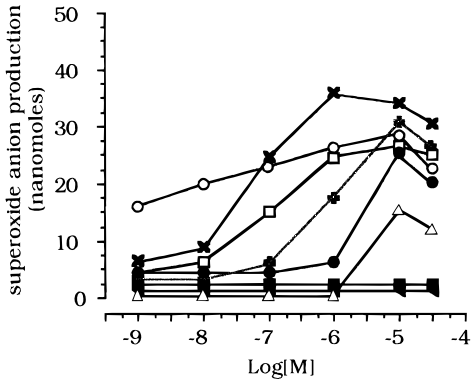

FIG. 4. Superoxide anion production of fMLP-OMe and its analogues toward human neutrophils. The points are the means of five separate experiments done in duplicate. SE are in the 0.1–4 nmol O_2^- range.

CONCLUSIONS

Chemotaxis. Acetylation is unable to elicit chemotactic activity on small peptides. Our results, furthermore, suggest that an elongation of the peptidic chain in the Met-Leu-Phe sequence could afford a biological response, as previously referred (*9*). As concerns formylated compounds, an alteration of the residue sequence strongly influences both the efficacy and potency of the derivatives.

Superoxide anion production is absent in acetylated tripeptides, but can be elicited from acetyl-tetrapeptides exclusively at concentrations higher than the control. All the formylated peptides are instead able to elicit a good biological response.

Lysozyme release. The behavior shown by acetylated tripeptides indicates that they are devoid of activity in this function too. Formyl-tetrapeptides are always efficacious

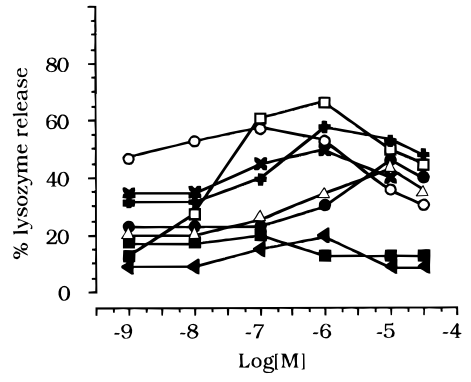

FIG. 5. Release of neutrophil granule enzymes evaluated by determining lysozyme activity induced by fMLP-OMe and its analogues. The points are the means of five separate experiments done in duplicate. SE are in the 1–6% range.

as secretagogue agents, while the corresponding acetyl-tetrapeptides seem to be sensitive to the sequence of the residues.

We can conclude that (i) acetyl-oligopeptides do not represent a useful tool for finding ligands able to fully activate human neutrophil responses, (ii) formyl-oligopeptides always, in some manner, elicit a sensitivity to the residue sequence in tripeptides, while tetrapeptides do not evidence this type of sensitivity.

Our results agree with both our previous studies (*14*,*15*) and present knowledge of the features of the amino-terminal pocket (*7*,*16*).

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