ON THE MECHANISM OF HUMAN POLYMORPHONUCLEAR LEUKOCYTE DEACTIVATION OF CHEMOTAXIS BY THE SYNTHETIC PEPTIDE FORMYL-METHIONYL–LEUCYL–PHENYLALANINE

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1. Introduction

Polymorphonuclear leukocytes respond chemotactically, in vitro, to a variety of compounds including denaturated proteins [1], factors derived from complement [2], bacterial products [3] and synthetic peptides, the most potent being the peptide formyl-methionyl–leucyl–phenylalanine (f-Met–Leu–Phe) [4,5]. In addition f-Met–Leu–Phe induces granule enzyme secretion and superoxide production when added with cytochalasin B to rabbit neutrophils in suspension [4,6] and aggregates these cells [7]. There is evidence that all these functions result from the interaction of the peptide with a common membrane receptor [4,6–8] which has been demonstrated, by direct binding studies, to exist on the cell surface of rabbit [9] and human neutrophils [10].

If rabbit peritoneal neutrophils were incubated with high concentrations of a chemoattractant derived from complement, then washed, the cells showed a diminished chemotactic responsiveness towards the same stimulus [11]. This phenomenon had been termed 'chemotactic deactivation' [11] and although it has been noted with other chemotactic factors, including f-Met–Leu–Phe [12] it has not received a satisfactory explanation. However, deactivation of neutrophils is not restricted to chemotaxis [13]. If rabbit peritoneal neutrophils are incubated with f-Met–Leu–Phe, then washed before being rechallenged with the same peptide and cytochalasin B, the cells show a decreased secretory responsiveness. This deactivation might be related to a loss of binding sites for the peptide [13].

Since in many other systems (reviewed [14]) deactivation (or desensitization or refractoriness) has been shown to be effectively related to a loss of binding sites on the cells for the effector, we have decided to see if this was true also for chemotactic deactivation.

Here we show, using human peripheral blood neutrophils, that there is a time and dose-dependent decrease in the chemotactic responsiveness of these cells towards f-Met–Leu–Phe when they are preincubated with the same peptide then washed. However, these changes in chemotactic responsiveness are neither accompanied by a loss of binding sites for this peptide nor by a difference in the affinity for its receptor.

2. Materials and methods

f-Met–Leu–Phe was purchased from UCB, Bruxelles. f-Met–Leu–[^{3}H]Phe was prepared by J.L.M. by catalytic dehalogenation of the peptide f-Met–Leu–Phe(I)-OH (Bachem, Switz.) and was shown to be 100% pure by thin-layer chromatography and amino acid analysis. Its specific activity was 24 Ci/mmol and it was biologically indistinguishable from unlabelled f-Met–Leu–Phe.

Polymorphonuclear neutrophils were obtained from heparinized peripheral blood of human volunteers by method in [10] and were ≥98% pure.

Neutrophils were washed twice and resuspended in solution A (Hanks' balanced salt solution buffered with 50 mM Hepes (pH 7.2) containing 2% bovine serum albumin) at 15×10^6 cells/ml and incubated, at 37°C, in solution A alone or in solution A containing different concentrations of the chemotactic peptide. At the end of the incubation period, the cells were collected by centrifugation ($350 \times g$ for 10 min) and

resuspended in 40 ml solution A. After 10 min at 37°C, the sedimentation and resuspension were repeated. After an additional 10 min at 37°C, the cells were again centrifuged for 10 min at 350 × *g* and resuspended in solution A. Final concentrations at the end of the washings were 10–12 × 10^6 cells/ml. Aliquots of this suspension of washed cells were used to study f-Met–Leu–[^{3}H]Phe binding or chemotaxis towards fresh f-Met–Leu–Phe.

Chemotaxis was measured by using ^{51}Cr-labelled neutrophils as in [15], in a modified Boyden chamber (Celloplex, Basel) with 2 cellulose nitrate filters of poresize 3 μm and 5 μm on top of each other. The ^{51}Cr-labelled neutrophils at 4 × 10^6 cells/ml were placed in the upper compartment of the chemotactic chamber. The chemotactic peptide was diluted, from a stock solution at 10^{-3} M in dimethylsulfoxide, in solution A and used in the lower compartment of the chamber at final conc. 10^{-8} M. The chambers were incubated for 3 h at 37°C in 100% humidity and 5% CO_2. At the end of the incubation the lower filters were removed, rinsed and counted in a γ counter (Kontron Electronics CG 4000). Results are expressed as cpm in lower filter/total cpm put in the upper compartment of the chamber.

All binding studies were performed in solution A at 22°C in plastic microcentrifuge tubes (Beckman) in 0.2 ml final vol. Neutrophils were incubated with increasing concentrations of f-Met–Leu–[^{3}H]–Phe for 30 min. The incubation was started by addition of cells and terminated by dilution with 1 ml ice-cold Hanks' solution followed by centrifugation (10 s at 12 000 rev./min). The supernatant was discarded by aspiration and the cell pellets were then washed rapidly with 1 ml ice-cold Hanks' solution and separated again by 10 s centrifugation. The cell pellets were then dissolved in aqueous sodium dodecyl sulfate (250 μl, 0.1%) and transfered to counting vials containing 10 ml Instagel. Radioactivity was measured in a liquid scintillation spectrophotometer (Kontron Electronics SL 3000). Non-specific binding was defined as the amount of binding not inhibited by 50 μM unlabelled f-Met–Leu–Phe and was usually 10–15% of the total counts bound. Specific binding was defined as the total amount of f-Met–Leu–[^{3}H]Phe bound minus the non-specific binding.

3. Results

Table 1 presents the results of experiments in which various suspensions of neutrophils were incubated at 37°C with f-Met–Leu–Phe or in solution A alone. After washing, the cells were tested for chemotaxis towards the same fresh peptide. There is a time- and dose-dependent inhibition of chemotaxis when the cells are preincubated with f-Met–Leu–Phe at $\geq 10^{-8}$ M.

After preincubation and washing, we also determined the peptide receptor concentration/cell by incubating the washed cells at 22°C, for 30 min (a time shown to be necessary to reach equilibrium) with increasing concentrations of f-Met–Leu–[^{3}H]Phe (fig.1). It can be seen that f-Met–Leu–[^{3}H]Phe binding to cells that had been preincubated with 10^{-8} M f-Met–Leu–Phe for 1 h, or with 10^{-6} M peptide for

Table 1
Effect of preincubation of neutrophils with f-Met–Leu–Phe on locomotion towards the same peptide

Cells migrating towards	Preincubation (min)	No peptide	Cells preincubated with f-Met–Leu–Phe				
			10^{-10} M	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
			(% cells migrating in the lower filter)				
No peptide	0	1.45	–	–	–	–	–
f-Met–Leu–Phe 10^{-8} M		8.66					
No peptide	5	1.51	1.61	1.63	1.58	1.55	1.53
f-Met–Leu–Phe 10^{-8} M		7.60	7.83	8.27	5.78	4.34	2.12
No peptide	15	1.63	1.58	1.51	1.43	1.33	1.61
f-Met–Leu–Phe 10^{-8} M		8.33	7.44	7.72	3.29	1.66	1.91
No peptide	30	1.93	1.55	1.93	1.46	1.55	1.74
f-Met–Leu–Phe 10^{-8} M		8.79	7.32	5.86	2.9	1.71	1.93
No peptide	60	1.52	1.61	1.34	1.55	1.37	1.58
f-Met–Leu–Phe 10^{-8} M		7.43	7.60	2.15	2.01	1.43	1.65

Each figure is the mean of 4 different determinations done in duplicate

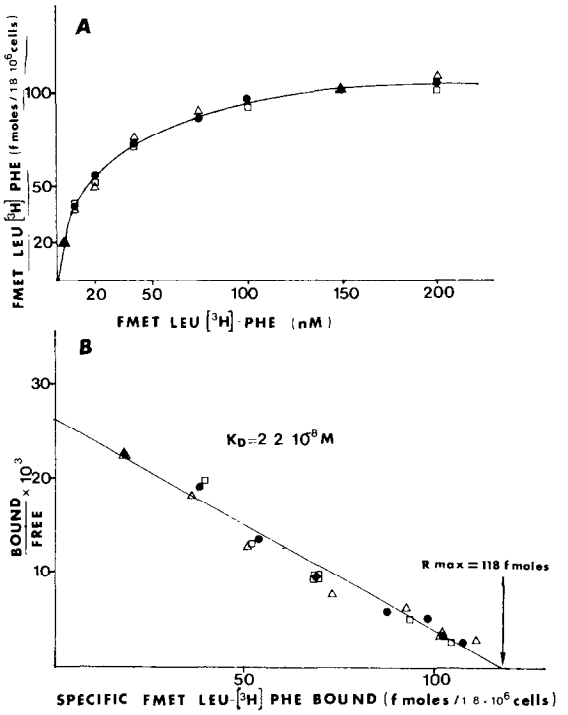

Fig.1. (A) Binding of f-Met–Leu–[^{3}H]Phe to neutrophils as a function of concentration of f-Met–Leu–[^{3}H]Phe. f-Met–Leu–[^{3}H]Phe at the indicated concentrations was incubated with human neutrophils for 30 min at 22°C and specific binding was measured (●——●). Cells which had been incubated for 1 h at 37°C in solution A alone. (□——□) Cells which had been incubated for 1 h at 37°C in the presence of f-Met–Leu–Phe 10^{-8} M and then washed. (△——△) Cells which had been incubated for 30 min at 37°C in the presence of f-Met–Leu–Phe 10^{-6} M and then washed. Each value shown represents the mean of 3 determinations done in duplicate. (B) The data are plotted according to Scatchard's analysis.

30 min, was identical to that of cells that had not been exposed to the peptide (fig.1A). Analysis of the Scatchard's plots (fig.1B) indicates that there is neither decrease in the effective concentration of receptors, nor alteration in affinity. With either set of cells, the equilibrium dissociation constant (K_d) was 2.2×10^{-8} M. At saturation, the 1.8×10^6 cells present in the incubation mixture are capable of binding 118 fmol f-Met–Leu–[^{3}H]Phe. This value corresponds to ~40 000 sites/cell is 20 times higher than that in [10]. This may be because the receptor sites were not fully saturated for the peptide on the neutrophils in fig.3 and fig.4A of [10]. Using rabbit peritoneal neutrophils and the radiolabelled analogue [^{3}H]f-NorLeu–Leu–Phe, [9] K_d and R_{max} of 1.5×10^{-9} M and 102 000 sites/cell, respectively were found; in [15] $K_d = 2 \times 10^{-8}$ M.

In further set of experiments we tested the ability of our washing procedure to reverse the binding of f-Met–Leu–[^{3}H]Phe. Cells (15×10^6) were incubated, in duplicate, with f-Met–Leu–[^{3}H]Phe 4×10^{-8} M in 1 ml solution A. After 1 h at 37°C, two 100 μl aliquots were removed, washed with ice-cold Hanks' solution and their radioactivity determined. The 800 μl left were diluted with 40 ml solution A at 37°C and washed twice as in section 2. At the end of the washing procedure, the cell pellet was resuspended in 1 ml solution A at 22°C. Two 100 μl aliquots were immediately removed, washed with ice-cold Hanks' solution and their radioactivity measured. The remaining cells were separated in three 190 μl fractions on which the total and non-specific binding of fresh f-Met–Leu–[^{3}H]Phe, 4×10^{-8} M, were measured as in section 2. Cells preincubated in solution A alone were treated under the same conditions. The results of these experiments are presented in table 2. It can

Table 2
Reversal of f-Met–Leu–[^{3}H]Phe binding by the washing procedure

Cell treatment	cpm/2 × 10^6 cells
Cells preincubated with f-Met–Leu–[^{3}H]Phe 4×10^{-8} M at 37°C for 1 h, washed at 0°C and counted	1834
Cells preincubated with f-Met–Leu–[^{3}H]Phe 4×10^{-8} M at 37°C for 1 h, washed twice at 37°C and counted	590
Cells preincubated with f-Met–Leu–[^{3}H]Phe 4×10^{-8} M at 37°C for 1 h, washed twice at 37°C and rechallenged with fresh f-Met–Leu–[^{3}H]Phe 4×10^{-8} M for 30 min at 22°C	1920
Cells preincubated and washed at 37°C as above and rechallenged with fresh f-Met–Leu–[^{3}H]Phe 4×10^{-8} M and f-Met–Leu–Phe 5×10^{-5} M for 30 min at 22°C	680
Cells preincubated in solution A alone for 1 h at 37°C, washed twice at 37°C and challenged with f-Met–Leu–[^{3}H]Phe 4×10^{-8} M for 30 min at 22°C	1433
Cells preincubated in solution A alone for 1 h at 37°C, washed twice at 37°C and challenged with f-Met–Leu–[^{3}H]Phe 4×10^{-8} M and f-Met–Leu–Phe 5×10^{-5} M for 30 min at 22°C	199

Each value is the mean of 2 different determinations done in duplicate. 1433 cpm/2 × 10^6 cells represent 80 fmol peptide bound on the cells

be seen that cells preincubated either in solution A alone or in presence of f-Met–Leu–[^{3}H]Phe can bind the same amount of fresh radiolabelled peptide corroborating the results of fig.1. It can be seen also that cells preincubated with f-Met–Leu–[^{3}H]Phe and then washed thoroughly at 37°C keep an amount of radioactivity slightly higher than the non-specific binding. This result and our data (not shown) on the kinetics of binding of f-Met–Leu–[^{3}H]Phe on human neutrophils suggest that a certain amount of peptide is interiorized after its association with the cells.

4. Discussion

We have demonstrated that human peripheral blood neutrophils preincubated with the synthetic chemotactic peptide f-Met–Leu–Phe and then washed lose, in a time and dose-dependent fashion, their ability to respond chemotactically to the same peptide. This deactivation of chemotaxis is already manifest after 5 min preincubation of cells with peptide at $>10^{-8}$ M.

Using radiolabelled f-Met–Leu–Phe we have shown that our washing procedure is able to displace all the peptide that was originally bound to the cells during the preincubation period. Thus neutrophils which had been preincubated with f-Met–Leu–Phe at $\leqslant 10^{-5}$ M and then washed have the same total binding capacity for f-Met–Leu–[^{3}H]Phe than do cells preincubated in buffer alone.

Our results are in accord with those in [16] (while this manuscript was in preparation) who also found that human neutrophils preincubated with high concentrations of f-Met–Leu–Phe are chemotactically deactivated. They also found that these deactivated cells do not respond chemotactically to a gradient of complement-derived factors. Since these chemoattractants (essentially C5a and C3a [17]) are totally unrelated to f-Met–Leu–Phe and have different specific receptors on the neutrophils [18] it is highly improbable that a loss of binding sites for f-Met–Leu–Phe would impair neutrophil chemotaxis towards the complement-derived factors. The explanation for chemotactic deactivation of human neutrophils claimed in [16] was that high concentrations of f-Met–Leu–Phe render the cells hyperadhesive leading to profoundly diminished directed and random migration.

Another explanation using human neutrophils and two chemoattractants totally unrelated to f-Met–Leu–Phe [12] was that deactivation is the functional manifestation of an unorganized chemotactic factor-sensitive microtubule assembly and not the bockade of available receptors. However, levamisole and the calcium ionophore A23187, if present during the preincubation of neutrophils with f-Met–Leu–Phe, could prevent the inhibition of chemotaxis [19]. Since neither drug interferes with the binding of f-Met–Leu–[^{3}H]Phe (our data not shown) it is possible that cyclic nucleotides or calcium, or both, may be involved in the molecular mechanism of neutrophil deactivation.

In conclusion, these data show, by direct binding experiments with f-Met–Leu–[^{3}H]Phe, that the chemotactic deactivation of human neutrophils, induced by preincubation of the cells with the peptide, is not due to a loss of binding sites for the chemoattractant. Whatever the molecular mechanism for this deactivation of chemotaxis, it certainly involves a step beyond the binding of the peptide to its membrane receptor. Work is in progress in our laboratory to investigate this problem.

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