DOSE-DEPENDENCE OF THE VARIOUS FUNCTIONAL RESPONSES OF NEUTROPHILS TO FORMYLPEPTIDES*

Activation, Regulation, and Inverse Effects According to the Agonist Dose and CellCondition

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1. Introduction

The up- and down-regulation of the response to a specific stimulatory compound, according to its doses and to the sensitivity of the cells, is a widespread process in biology, immunology and endocrinology. Leukocytes are particularly suitable models for studying these changes of biological response as these cells may be easily isolated from blood and from inflammatory exudates and appear to be regulated by a large variety of mediators both *in vitro* and *in vivo* (disease states).

Neutrophil granulocytes are one of the main cell types involved in the first defense lines against infections and in the acute inflammation. Following the local or systemic generation of membrane perturbing agents and biological signals such as cytokines, complement factors and bacterial products, neutrophils undergo a series of highly regulated functional modifications. The expression and activation of specific membrane glycoproteins induce cell adherence to endothelium or to subendothelial structures, thus allowing the leukocyte extravasation and the chemotactic movement into the connective tissue.

These cells are endowed with a powerful armamentarium of enzymes and antimicrobial peptides. Moreover, upon activation, they produce a huge amount of excited oxygen species, including superoxide anion (O2-) and its derivatives, that participate in the microbial killing but also in the possible tissue damage caused by the dysfunction of neutrophil activation in a number of pathologies (Weiss, 1989; Smith, 1994). Since the duration and the magnitude of the functional responses are important factors in determining the final balance - positive or unfavourable for the host - of neutrophil activation, these phenomena are subject to a fine and complex regulation at the level of receptors, transduction mechanisms and effector enzymes.

Here we summarize a series of studies carried out in our laboratory with the aim of characterizing the sensitivity limits of normal and primed human neutrophils to the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP). We studied: a)

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the oxidative metabolism, assessed as the burst of O2- production, b) the adhesion to serum-coated surfaces, c) the release of lysozyme, a bactericidal enzyme, d) the actin polymerization, a rapid agonist-stimulated response of the cell cytoskeleton, e) the expression of fMLP receptors, f) the activation of two important intracellular biochemical changes that follow receptor stimulation, i.e. free Ca2+ increase and cyclic AMP (cAMP) increase.

These data are of interest both for an understanding of neutrophil physiology in the context of the inflammatory reactions and, in a wider perspective, for the understanding of the complex events involved in the changes of sensitivity and of responsiveness of the cells treated with different doses of receptor agonists. Several experiments where the phenomena of direct activation, priming, desensitization, and reverse effects have been observed are reported.

2. Dose-response Curves of Activation, Priming and Desensitization

The dose-response curves of the effect of increasing doses of fMLP on O2- production and on adhesion of human blood neutrophils are shown in figure 1.

The left panels of the figure (A and C) show the *direct* stimulatory effect of the agonist: O2- production was triggered by fMLP doses of 10 nmoles (nM) and higher, reaching a maximum at 100 nM, while adhesion required at least 30 nM fMLP and increased up to 1000 nM. The right panels of the figure (B and D) show the net effect of a *second addition* of high doses of fMLP (100 nM) to the cells that had been previously treated with the range of fMLP concentrations indicated in the X axis of the figure. It can be seen that the response of neutrophils pre-treated with doses ranging from 0.1 to 5 nM (i.e. sub-stimulatory doses) was higher than that of neutrophils not pre-treated with fMLP, both in terms of O2- production (B) and in terms of adhesion (D). Therefore, low doses of the peptide primed the cells to a subsequent higher response to the same agent, a phenomenon that we called *homologous priming* (Bellavite *et al.*, 1993a). Figures 1B and 1D also show that the response of neutrophils pre-treated with doses ranging from 10 nM to 1000 nM (i.e. stimulatory doses as shown in the left panels of the figure) had a decreased response both in terms of O2- production and in terms of adhesion. In other words, the stimulatory doses of the peptide *desensitize* the cells to a subsequent challenging with the same agent, and the extent of the desensitization is proportional to the extent of the previous stimulation. Similar dose-dependent stimulatory, priming and desensitising responses were observed by measuring the release of lysozyme.

Priming induced by low doses of fMLP is not only homologous but also heterologous, because we and others have observed that pretreatment with fMLP increases also the response to other unrelated agents, such as phorbol-myristate acetate (PMA), the active principle of croton oil. On the other hand, the desensitization induced by high doses of fMLP is exclusively homologous, because the response to PMA was not inhibited but, instead, was further augmented (Table 1).

3. Other Dose-dependent Phenomena

We then investigated the possible mechanisms underlying these low-dose (priming) and high-dose (direct activation and homologous desensitization) effects, by looking at the correlation between these phenomena and other fMLP-induced biochemical events.

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DOSE DEPENDENCE OF NEUTROPHIL RESPONSE

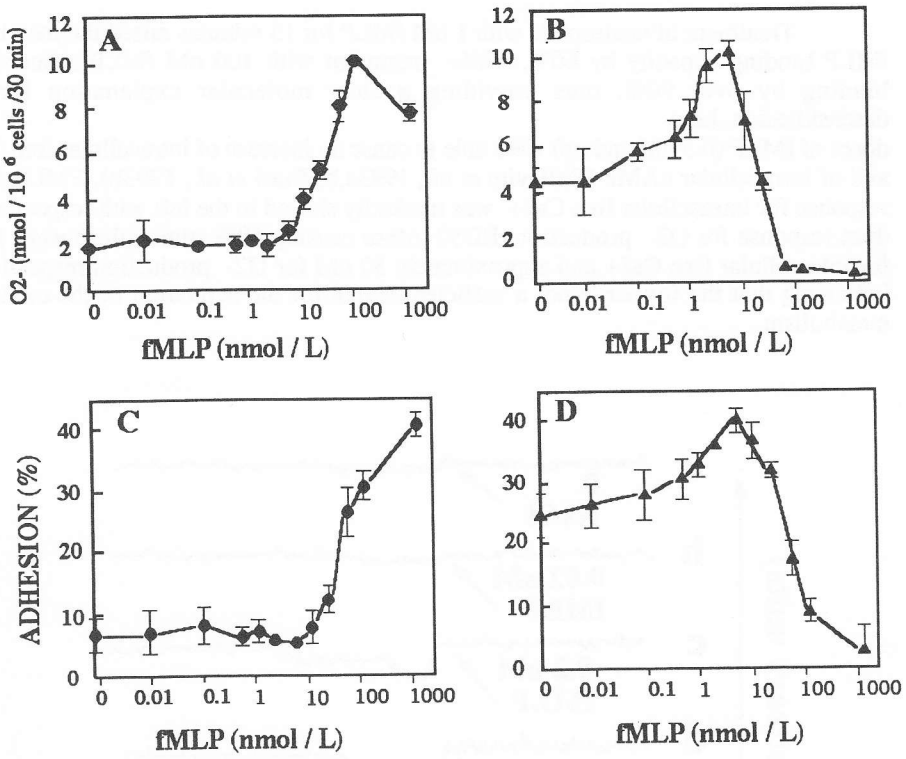

Figure 1. O2- production (A, B) and adhesion (C, D) of human neutrophils stimulated with different doses of fMLP. A, C: direct stimulation; B, D: effect of 100 nM fMLP on cells pretreated for 15 minutes with the indicated doses of fMLP. Blood neutrophils were obtained from healthy subjects by centrifugation of EDTA-anticoagulated blood over Percoll gradients (Metcalf *et al.*, 1986). O2- was measured by the reduction of ferricytochrome c in multiwell microplates and adherence to the serum-coated surface of microplates was quantitated by acid phosphatase assay (Bellavite *et al.*, 1992). Values are mean ± S.D. of triplicate determinations from a representative experiment of 12 performed.

TABLE 1. Effect of 15 minutes of pretreatment with different doses of fMLP on the O2- production induced by fMLP and PMA

Preincubation	O2- production (nmoles/106 cells/30 min)	
	Second stimulus fMLP (100 nM)	Second stimulus PMA (10 ng/ml)
Buffer (control)	4.1 ± 0.5	13.6 ± 0.3
5 nM fMLP	10.1 ± 0.9	18.4 ± 1.3
100 nM fMLP	0.4 ± 0.4	35.5 ± 2.2

Treatment of neutrophils with 1 nM fMLP for 15 minutes raised the membrane fMLP binding capacity by 60%, while treatment with 100 nM fMLP reduced the binding by over 90%, thus providing a clear molecular explanation for the desensitization. Low
doses of fMLP (0.5 nM and up) were able to cause an increase of intracellular free Ca2+ and of intracellular cAMP (Bellavite *et al.*, 1993a,b; Biasi *et al.*, 1993b). FMLP dose-response for intracellular free Ca2+ was markedly shifted to the left with respect to the dose response for O2- production: ED50 (dose causing 50% stimulation) were 1 nM for intracellular free Ca2+ and approximately 50 nM for O2- production respectively, indicating that the former is not a sufficient signal for the activation of the oxidative metabolism.

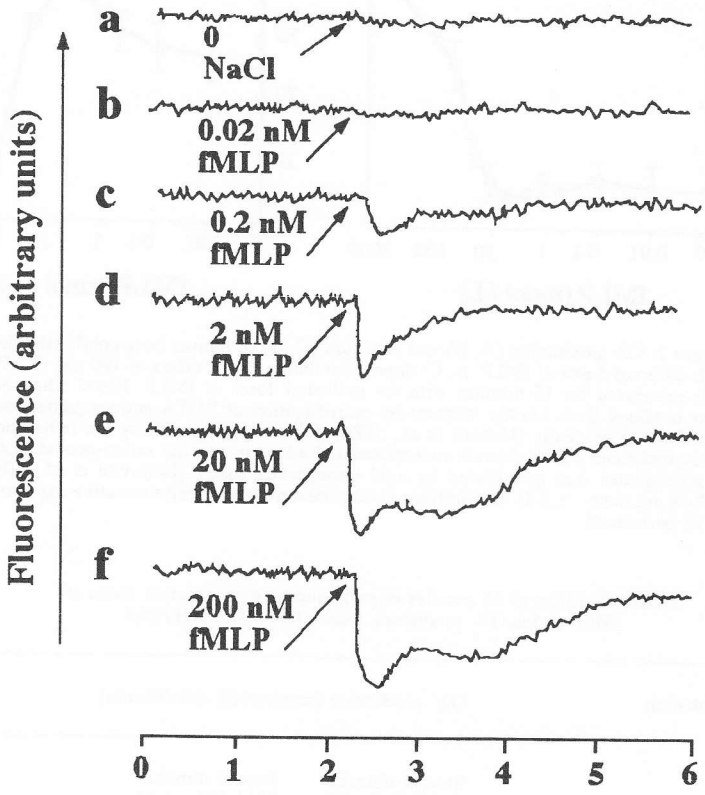

Figure 2. Recording traces of right-angle light scatter response (actin polymerization) of neutrophils to fMLP, assessed fluorimetrically according to Omann *et al.* (1989).

These data may indicate that the priming effect is the result of an increase in the number of receptors or of other mechanisms of post-receptor regulation, particularly linked to the increase of intracellular Ca2+. It should be noted, however, that many models of priming exist and that an increase of Ca2+ does not appear to be an absolute requirement (Biasi *et al.*, 1993b). By no means extraneous to priming are other biological events occurring at post-receptor level, such as the phosphorylation-dephosphorylation of specific proteins, the state of assembly of cytoskeletal proteins, and the constitution of membrane lipids. The latter point appears to be particularly relevant for the *in vivo* regulation of neutrophil responsiveness, as documented by our recent findings that the O2- release in response to fMLP is positively correlated with the cellular content of arachidonic acid and inversely correlated with linoleic acid and palmitic acid content (Bellavite *et al.*, 1995). These results suggest that the fatty acid composition of blood neutrophils may be a critical factor determining the capability of releasing free radicals in response to formylpeptides and are of relevance in view of a possible manipulation of the lipid composition of cell membranes by diet changes.

Another cell response that appeared to be sensitive to very low doses of fMLP was the actin polymerization, that is associated with cytoskeletal and morphological changes of the cells (figure 2). Doses of fMLP as low as 0.2 nM triggered a rapid actin polymerization response, that was already maximal at 20 nM. Of particular interest are also the time-dependent oscillations of scattering, induced by medium-high doses of fMLP, a finding in agreement with previous ones (Omann *et al.*, 1989).

4. Inverse Effects of Different fMLP Doses on Neutrophil Adhesion

We then studied the metabolic and adhesion responses to fMLP of neutrophils which were treated with bacterial endotoxin (lipopolysaccharide, LPS) or which were harvested from in a *in vivo* experimental inflammation (24-h exudation through a skin-window procedure).

Both LPS treatment and inflammation enhance the neutrophil's oxidative burst in response to fMLP, i.e. they induce cell priming (table 2). Moreover, by investigating the dose-response relationships of the adhesion response in these primed cells, we have observed an unexpected phenomenon (table 2 and figure 3): a) priming augments cell adhesion to serum-coated culture wells in the absence of further stimulation; b) addition of low, substimulatory doses of fMLP (0.5 nM to 5 nM) inhibits and reverses the spontaneous adhesion, c) high fMLP doses (> 100 nM) increase the adhesion and are additive to the spontaneous adhesion induced by priming. In conclusion, the chemotactic agent fMLP, which is considered to be an activator of neutrophil adhesion, paradoxically inhibits the same cell response at low doses when used in primed cells.

5. Discussion

We have investigated several different neutrophil functional activities triggered by contact with chemotactic peptides. The evidence here reported demonstrates that "early" responses of neutrophils to low doses of fMLP involve both structural (actin polymerization) and biochemical (Ca2+ and cAMP increase) changes, which are associated with inhibition of adhesion, while adhesion and superoxide release should be

TABLE 2. A comparison of the effects of low doses and high doses of fMLP on O2- production and adhesion of normal and primed neutrophils

Source of neutrophils	O2- production (nmoles/106 cells/30 min)		
	No fMLP	Low-dose fMLP[a]	High-dose fMLP[b]
Blood	0.6 ± 0.6	1.6 ± 1.0	11.5 ± 3.0
LPS-primed	3.6 ± 0.3	2.8 ± 0.3	18.9 ± 1.7
Exudate-primed	0.8 ± 1.0	5.4 ± 2.0	23.9 ± 5.3
	Adhesion (% of total)		
	No fMLP	Low-dose fMLP[a]	High-dose fMLP[b]
Blood	5.6 ± 3.4	3.7 ± 1.6	27.5 ± 8.1
LPS-primed	20.9 ± 10.5	7.4 ± 6.1	40.3 ± 16.9
Exudate-primed	17.1 ± 10.3	8.4 ± 4.9	39.2 ± 14.7

Exudate neutrophils were isolated according to the skin-window method described by Senn, with modifications (Biasi *et al.*, 1993a). LPS-primed neutrophils were obtained by incubating blood neutrophils for 1h with 1µg/ml LPS. a,b: Low doses 1 nM to 5 nM, high doses 100 nM to 500 nM, according to different experiments. The table summarizes data from experiments reported in Bellavite *et al.* (1993b) and Bellavite *et al.* (1994). The values significantly different ($p < 0.01$, paired Student t test) from the respective values obtained in the absence of fMLP (no fMLP) are shown in bold.

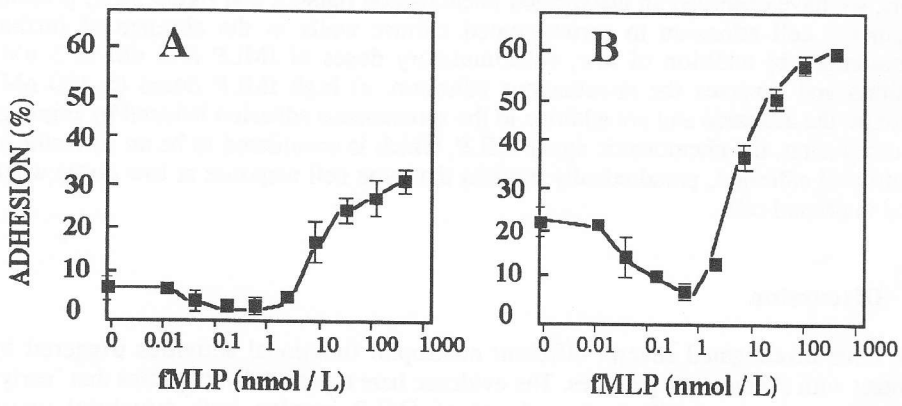

Figure 3. Effect of different doses of fMLP on the adhesion of human neutrophils. A: blood cells, B: cells isolated from a skin-window inflammatory exudate of the

considered as "late" or "extreme" responses to chemotactic factors, which require a 100-fold higher dose of this stimulant.

These results indicate that actin polymerization, a typically non-linear cellular phenomenon, reflects one of the earliest cellular events, occurring at very low agonist doses. Our data are in agreement with those reported by others, showing that half-optimal O2- production requires the occupancy of about 30% of the fMLP receptors, while intracellular Ca2+ increase and actin polymerization require occupancy of less than 3% and 0.1% receptors respectively (Sklar *et al.*, 1985).
A summary of the various responses which have been investigated and of their dependence on the fMLP dose is reported in table 3.

TABLE 3. Effects of different doses of fMLP on various neutrophil functions

Cell function	Low doses (0.1 to 5 nM)	High doses (10 to 1000 nM)
Actin polymerization	Activation	Activation and oscillations
Intracellular free Ca2+	Increase	Increase
Intracellular cAMP	Increase	Increase
fMLP receptors	Increase	Decrease
Adhesion (normal blood cells)	No effect	Activation
Adhesion (primed cells)	Inhibition	Activation
O2- production	No effect	Activation
Lysozyme release	No effect	Activation

These findings provide a reproducible *in vitro* model of the complex biological events occurring in leukocytes when they are treated with different doses of a bacterial product. In a wider perspective, these findings provide an example of how biologically active compounds may cause inverse effects on a system endowed with regulatory (feed-back) controls, when either the *doses* of the compound, or the *sensitivity* and the *responsiveness* of the system are varied by changes of environmental conditions. Neutrophil adhesion is the result of a fine regulation that enables the cell to move onto a surface by continuous adhesion/detachment events. Our current hypothesis is that low doses of fMLP cause inhibition of cell adherence because they increase cellular cAMP. In fact, adhesion is inhibited by adenosine, a physiologically relevant agent that increases intracellular cAMP (Bellavite *et al.*, 1992). Moreover, the adhesion of primed cells was inhibited also by addition of the membrane permeating cAMP analogue dibutirryl cAMP (1 mM) and of theophyllin (2 mM), which blocks the cAMP phosphodiesterase (Chirumbolo, unpublished observation).

The increase of adhesion and the oxidative burst are induced by much high doses of fMLP: it is conceivable that in order to trigger these responses, other intracellular messengers, besides cAMP and calcium, are required. Candidate molecules mediating the high-dose-dependent activation are diacylglycerols generated by phospholipid breakdown, that are activators of protein kinase C and whose production requires high fMLP doses (10 to 1000 nM) (Dougherty *et al.*, 1989).

Our data have shown the four different states of activation in neutrophils: a) resting state (dormant cells); b) homologous and heterologous priming, with increased O2- and decreased adhesion responses; c) full activation, where the cells are treated with high doses of agonists; d) specific desensitization to a second homologous stimulus. These types of tests are not simple laboratory artefacts, but allow us to reproduce a situation which occurs *in vivo*, i.e. where the cells in patients presenting bacterial infections or systemic inflammations are modified with respect to the cells in healthy people (Bloomfield and Young, 1988; Trautinger *et al.*, 1991; Smith, 1994). The fact that the disease conditions cause changes in receptor and transduction system sensitivity in various cells of the body is well known in many fields of medicine.

The concept is emerging that the leukocytes are involved in the cybernetic information networks of inflammation in a highly sophisticated and complex way: early activation of specific transduction systems, shape change, cell detachment from the stores, priming and chemotaxis require the occupancy of a minimum number of receptors, while the killing armamentarium (release of granule constituents, oxygen radicals) and the potentially harmful cell adhesion and spreading are triggered only when a massive engagement of membrane receptors is achieved. Finally, the effects of endotoxin and of the cellular lipid composition point to the existence of multiple control mechanisms and subtle synergisms between endogenous and exogenous compounds. All these factors are in connection with the individual's general state of health (infections, neuro-immuno-endocrine equilibrium, nutritional status).

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