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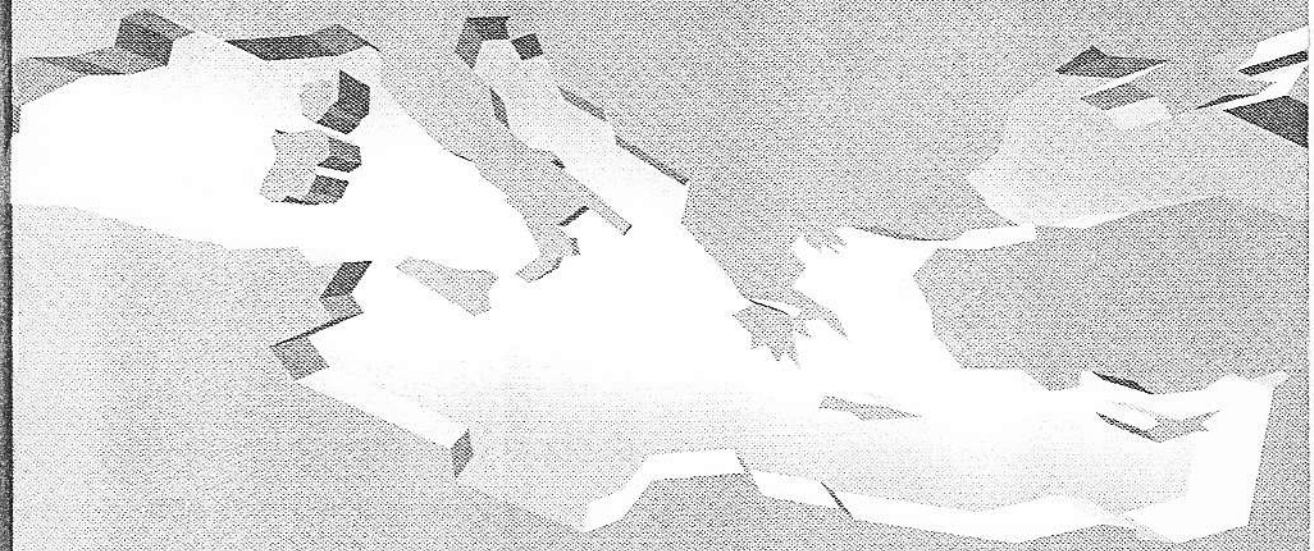

FIRST INTERNATIONAL CONGRESS:
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editrice compositori

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Istituto Superiore di Medicina Olistica e di Ecologia

UNIVERSITY OF URBINO (PS) - ITALY
September 24-27, 1992

Editrice Compositori

Nonlinear dose-dependent metabolic and adhesive responses of human neutrophils to chemotactic agents

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INTRODUCTION

This paper presents a condensed account of our recent studies regarding the development and applications of laboratory models for the regulation of neutrophil functions in response to agonists and antagonists. The function and pathology of leukocytes is of obvious interest from the standpoints of immunology and cell biology, but may also be of interest for the investigation of tne basic principles regulating the response of living organisms to low doses and ultra low doses of natural compounds. In this context, the study of these highly reactive and sensitive cells may provide new insights in the field of homoeopathy, where the problems of doses and individual sensitivity receive very considerable attention.

Homoeopathy is a global, integrated approach to human health and disease, which is open to discussion, there being a whole series of unresolved questions regarding both its efficacy and its supposed action mechanism(s). However, several *specific aspects and problems* may be investigated using the current molecular paradigm and biochemical or physical methods. In other words, while it is conceivable that homoeopathic issues cannot be fully understood from a biochemical standpoint alone, biochemistry and biology may help to provide answers to specific questions.

Since homoeopathy right from the onset has always claimed to act by regulating endogenous defense mechanisms, and inflammation/immunity is the main body defense and healing system, studies of leukocytes may be highly significant due to the widespread involvement of these cells in inflammatory reactions. Therefore, *in vitro* and *ex vivo* testing may provide important clues for investigation of homoeopathic drugs and homoeopathic principles by directly examining possible effects on cell function. This conclusion is supported by a substantial body of experimental data, reported in recent reviews by ourselves[1,2] and other groups[3-8]. «An explanation of the activity of homoeopathic preparations might be found more readily if cellular or animal models could be developed for their investigation»[8].

The laboratory models that we have developed deal with two main points: *a*) the problem of *doses*, i.e. the sensitivity limits of cell cultures to low doses, ultra low doses, or «high dilutions» of agonists, antagonists and other biologically active agents;
b) Hahnemann's traditional «*similia principle*», according to which compounds that cause disease symptoms in healthy subjects can heal patients presenting the same symptoms as a consequence of acquired disease[9].

Since inverse effects are the main topic of this paper, a definition of this concept is necessary. Inverse effects may be found both clinically and experimentally. Clinically, inverse effects may occur when *similar compounds cause pathological phenomena in healthy subjects and therapeutic effects in diseased subjects presenting the same or similar pathological phenomena*; this is a reelaboration in modern terms of the old «*similia similibus curentur*», proposed by C.F.S. Hahnemann two centuries ago.

Experimentally, inverse effects may be found when compounds *that are known as stimulators behave as inhibitors (or inhibitors behave as stimulators) of the same system when its sensitivity or responsiveness is changed according to variable experimental conditions.*

The data presented here derive from experiments designed to investigate the modulation of neutrophil functions during inflammation and during *in vitro* simulation of inflammatory conditions. These data are of interest both for an understanding of neutrophil biochemistry and for the application of the «similia principle» at cellular level.

Neutrophil granulocytes are one of the main cell types involved in the first defense lines against infections. These cells are endowed with a powerful armamentarium of enzymes and are also capable of producing and releasing huge amount of toxic oxygen derivatives that may serve for bacterial killing.

Granulocytes have a number of functions which can be evaluated with several laboratory methods. We have developed a sensitive and reliable assay system for the evaluation of superoxide production and adhesion[10]. Both these functions are dependent on external stimulation (Fig. 1): the metabolic activation and expression of adhesive anchoring systems follows membrane stimulation by factors of bacterial origin or factors produced by the inflammatory reactions.

The response of these cells to a specific stimulatory compound varies according to the sensitivity of the cells. Enhanced responses (priming) may be induced when neutrophils are pretreated with low doses of cytokines or bacterial products; dampened responses (desensitization, or adaptation) may be observed when the cells are pretreated with high doses of agonists acting on the same or related receptors as the second stimulant. Depression of leukocyte functions may also be found in a number of genetic or acquired diseases, the latter being due to inhibiting bacterial toxins, viruses or even endogenous factors.

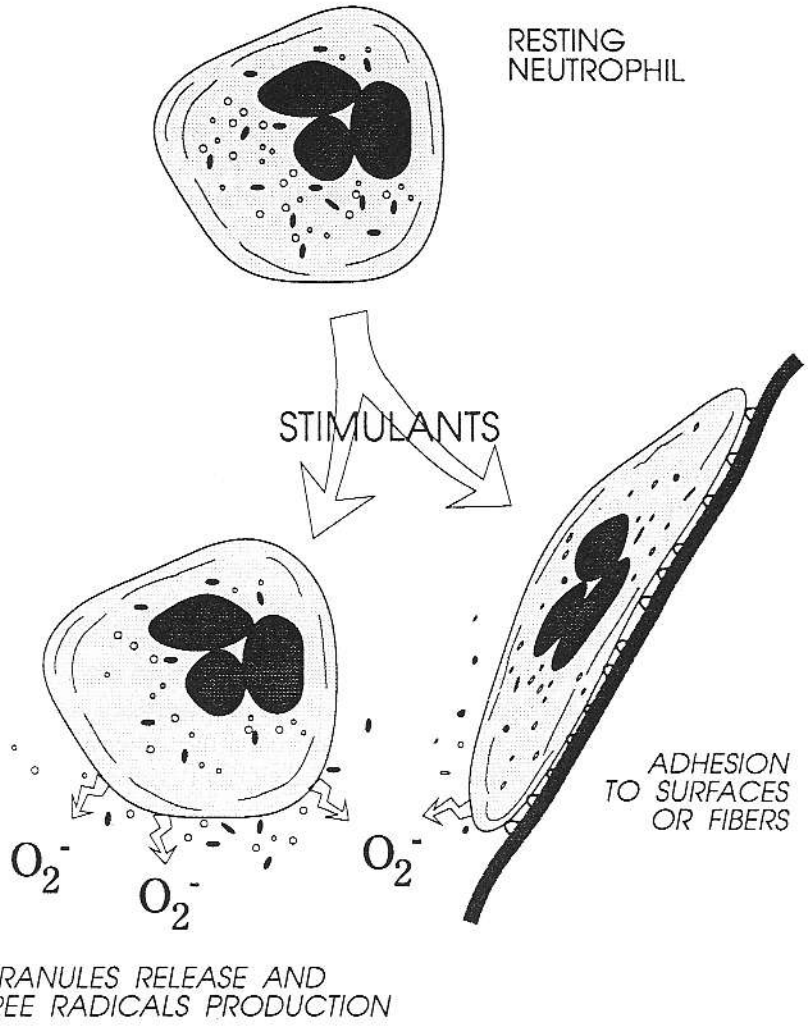

Figure 1 - Three main functions of an activated neutrophil granulocyte. Addition of suitable particulate or soluble stimulants induces granule release (exocytosis), activation of NADPH-dependent superoxide production and adhesion to surfaces or to other cells.

Therefore, human blood granulocytes are good models for the study of inverse effects because: *a*) they have receptors for and are highly responsive to a variety of endogenous and exogenous compounds; *b*) their functional activity may be up and down-regulated both *in vitro* (cytokines, bacterial products) and *in vivo* (disease states) and *c*) these cells may be easily isolated in both healthy and diseased subjects.

MATERIALS AND METHODS

Materials

The chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) and lipopolysaccharide (LPS, from *Escherichia coli* Serotype 026:B6) were purchased from Sigma Chemical Company, St. Louis, MO; fMLP (10^{-4} M) was dissolved in dimethylsulfoxide and stored at -20°C; cytochrome c was from Boehringer, Mannheim, Germany; purified human albumin from Behring Institut, Marburg, Germany; fetal bovine serum (FBS) from Flow Laboratories. FBS was inactivated by incubation at 56°C for 30 min, divided into aliquots, stored at -20°C and thawed before use.

Percoll was from Pharmacia, Uppsala. Sterile 96-well microtiter plates with flat-bottomed wells (Linbro type) were from Flow Laboratories; the microplates were pre-coated with FBS in order to abolish nonspecific cell activation. One hundred μl of 50% FBS in phosphate-buffered saline were dispensed into each well and the plate was incubated for at least 2 h at room temperature. Immediately before use, the plates were washed three times with 0.9% NaCl using an automatic plate washer (Easy Washer 2, SLT Labs Instruments).

Hank's balanced salt solution (without calcium and magnesium) was from Gibco Ltd, Paisley, Scotland. Other materials and reagents were of the highest purity available. In order to avoid contamination, a possible cause of artifactual activation or priming of the cells, sterile solutions and disposable plastic ware were used in all the experiments, which were carried out, whenever possible, under a laminar flow hood. Reagents were prepared using pyrogen-free water or 0.9% NaCl solutions.

Isolation of neutrophils

Human blood neutrophils were prepared from ethylene diamine tetraacetate-anticoagulated blood by centrifugation over Percoll gradients[11]. Cells (> 95% pure neutrophils) were finally suspended in a medium composed of Hank's balanced salt solution containing 5 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.2% human albumin (H-GCMA) at the concentration of 3×10^6/ml and kept at room temperature until use.

Superoxide anion production.

Superoxide anion was measured by the SOD-inhibitable reduction of ferricytochrome c[12] with a modification of microplate-based assays[10, 11]. The 96-well microtiter plates were prepared according to various schemes and combinations, depending on the test assay to be carried out (e.g. various incubation times or various concentrations of test compounds, etc.) and on the number of compounds to be tested. 1.5×10^5 cells were added to triplicate wells of fetal-bovine-coated

96-well microplates, in a final volume of 150 μl of assay medium composed of H-GCMA containing 0.15 mM cytochrome c as probe for O_2^- detection.

The plates were brought to 37°C in a humidified thermostat by preincubation for 15 min, then incubated at 37°C throughout the experiment. When indicated, the plates were rapidly transferred into a microplate reader (Reader 400, SLT Labs Instruments) and the reduction of cytochrome c was measured at 550 nm using 540 nm as the reference wavelength to avoid interference due to light scattering.

Adhesion

For adhesion measurements, immediately after reading cytochrome c reduction, the plates were transferred to an automatic washer (Easy Washer 2, SLT Labs Instruments) and subjected to two washing cycles with phosphate buffered saline at room temperature. Adherent cells were quantitated by measuring membrane enzyme acid phosphatase at 405 nm and the percentage of adhesion was calculated on the basis of a standard curve obtained with known numbers of neutrophils[10].

Receptor assay

Receptors for fMLP were quantitated using radiolabeled formyl-methyionylleucyl-(^{3}H)phenylalanine (NEN-Du Pont, Florence, Italy), as described by Metcalf *et al.*[11]. Binding was performed at 4°C for 30 minutes (saturation time) using the final concentration of 10^{-7} M radioactive ligand and 10^{-5} M nonradioactive (cold) ligand when necessary for assessing nonspecific and displaceable binding.

RESULTS

We investigated the response of neutrophils to chemotactic peptides with an assay for the superoxide production and for the adhesion capability of human neutrophils exposed to low and very low doses of agonists and inhibitors. The general purpose of our studies was to verify the limits of sensitivity and the regulating factors of isolated cells under standardized conditions.

Previous experience[10, 13] had shown that the optimal dose of the chemotactic agent fMLP for the triggering of superoxide production by human blood neutrophils is about 10^{-7} M (in homeopathic terms, a dilution between D7 and D8). Doses under 10^{-8} M were totally ineffective. We used two different methods for diluting the agonist, i.e. simple dilution and dilution followed by vigorous stirring (20 seconds with a Vortex-type mixer), but the dose-response curves obtained with the two methods did not differ appreciably. We pointed

out in a previous study[13] that these experiments have to be considered a first attempt to study the influence of dilution methods on neutrophil activation and do not rule out the possibility that significant effects of even higher dilutions may be obtained by changing experimental protocols (e.g. methods of dilution/succussion) or assay methods (e.g. using more sensitive methods). Only further experience will clarify this important point.

We then considered the possibility of increasing cell sensitivity by priming the cells with repeated treatment with active compounds. Priming is defined as an increase in activity exhibited by cells that have been pretreated with substimulatory doses of various compounds for which cells have specific receptors. The results achieved with various combinations of priming (i.e. pretreatment) and stimulatory (i.e. second addition) agents capable of exhibiting the priming effect in neutrophils have been reported[13-17].

An experiment showing the priming phenomenon is given in Table 1, where the extent of superoxide release is reported in relation to the concentration of fMLP. Neutrophils were treated for 1 hour with 1 μg/ml lipopolysaccharide from *E. coli* (endotoxin, LPS), various doses of fMLP were then added and the burst of superoxide release in the following 10 minutes was quantitated. It can be seen that treatment with endotoxin increases the cell metabolic activity triggered by fMLP as compared to normal cells. However, the potentiation of the respiratory burst by LPS was related to the extent of superoxide production and not to the sensitivity to low doses. Maximum fMLP stimulatory dose (peak) and minimum effective dose were both similar in treated and untreated cells. This evidence may indicate that in these experimental conditions priming is not due to enhanced receptor sensitivity but rather to the enhanced «responsiveness» of the cells to a given dose. Similar conclusions have been drawn from an *in vivo* priming model consisting in experimentally-induced skin-window inflammatory exudates[18].

The adhesion of the cells to serum-coated surfaces was then investigated in primed and normal cells. The fMLP doses that stimulate adhesion are similar

TABLE 1 - *Superoxide production by normal and LPS-treated neutrophils*

Stimulant	O_2^- production (nmol/10 min/10^6 cells)	
	Control cells	LPS-treated cells
None	0.3 ± 0.4	3.6 ± 0.3
10^{-9} M fMLP	0.2 ± 0.3	2.8 ± 0.3
10^{-8} M fMLP	2.2 ± 0.3	7.8 ± 0.8
10^{-7} M fMLP	7.2 ± 0.2	18.9 ± 1.7
5×10^{-7} M fMLP	4.2 ± 0.2	16.5 ± 0.6

Values are triplicate determinations ± SD.

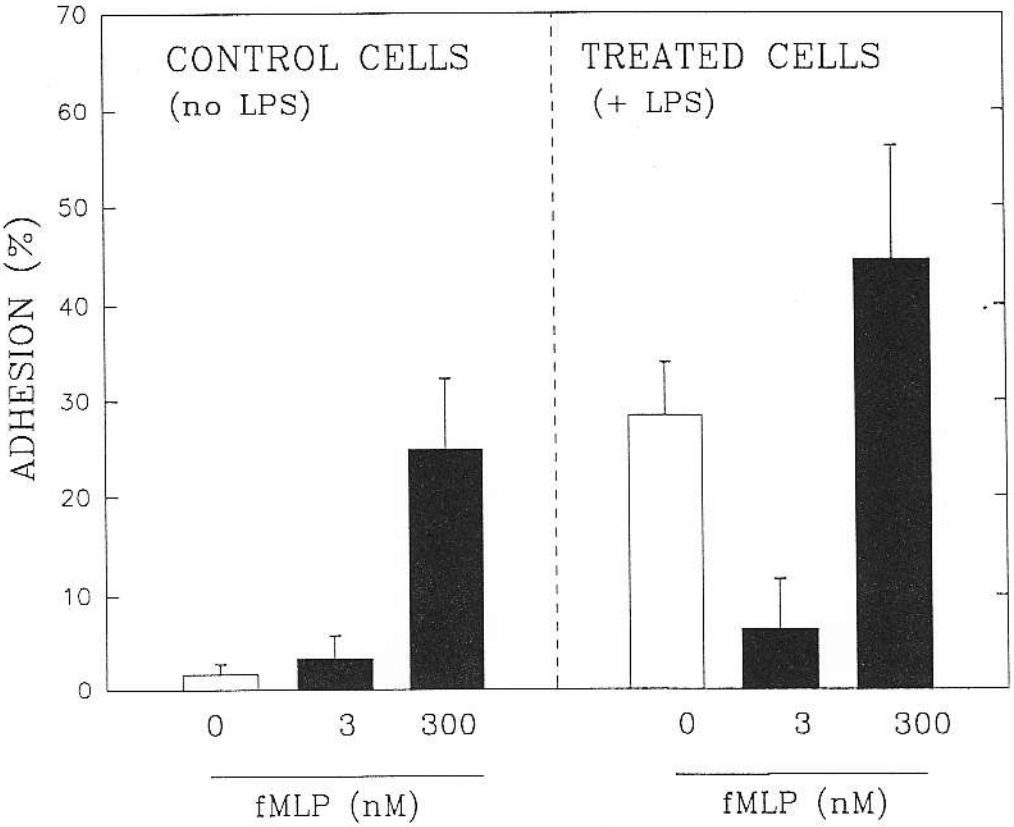

Figure 2 - Effect of different doses of fMLP on adhesion of LPS-treated and normal human blood neutrophils. Neutrophils were treated in suspension with and without 1 μg/ml LPS, then incubated for 40 minutes in microplates with the indicated fMLP concentrations. The number of adherent cells was quantitaled as described in Methods. Values are mean ± SD of 3 experiments.

to, or slightly higher than the doses capable of stimulating superoxide formation[10]. However, when studying adhesion, we noted (Fig. 2) that LPS-treated cells showed enhanced adhesion even in the absence of fMLP, and that, on addition of *low* doses of fMLP, the LPS-mediated adhesion was inhibited. In synthesis, the results reported in fig. 2 indicate that: *a*) untreated cells show only minimal adhesion to serum-coated plastic surfaces and this adhesion is not significantly affected by low doses (3 nM) of fMLP; *b*) fully activatory doses (300 nM) of fMLP induce a significant increase in cell adhesion; *c*) pretreatment of the cells for 1 h with 1 μg/ml endotoxin augments adhesion in the absence of further stimulation; *d*) addition of very low doses of fMLP (3 nM) inhibits the adhesion of endotoxintreated cells and *e*) high fMLP doses are additive to endotoxin in promoting adhesion.

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 (details of these and related experiments are reported in a manuscript in preparation). We repeated these assays six times with similar results.

In the course of studies designed to investigate the mechanisms of priming, we planned an experiment in order to evaluate the effect of repeated additions of the same chemotactic peptide fMLP. Previous papers had shown that pretreatment of the cells with the chemotactic peptide fMLP prevents further activation by a second dose (receptor-specific desensitization, or adaptation). Adaptation has been recognized primarily as a homologous phenomenon: that is,

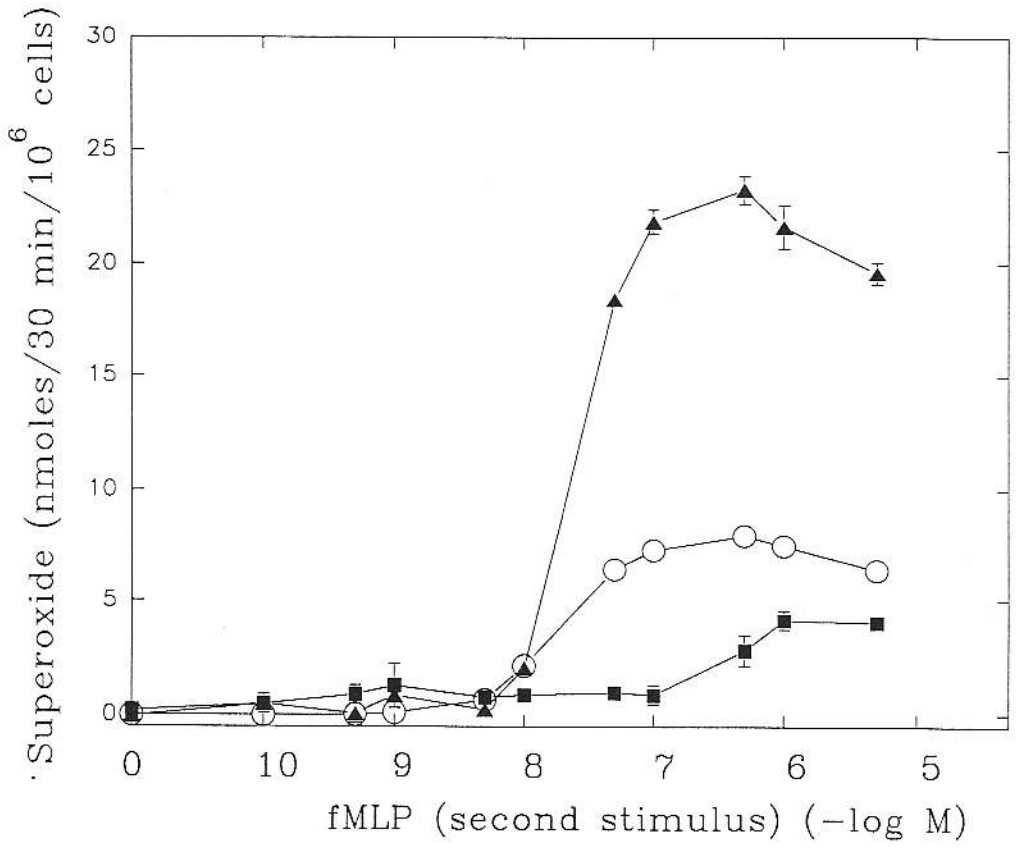

Figure 3 - Dose-response curves of human neutrophils stimulated with fMLP after pretreatment with various doses of fMLP. Neutrophils were preincubated for 15 minutes with no fMLP (circles), with high fMLP doses (10^{-7} M, squares) and with low fMLP doses (5×10^{-9} M, triangles), and then treated with a second dose of fMLP, as shown on the x axis of the figure. Superoxide release was quantitated by the cytochrome c assay described in Methods. Values are mean ± SD for triplicate assays.

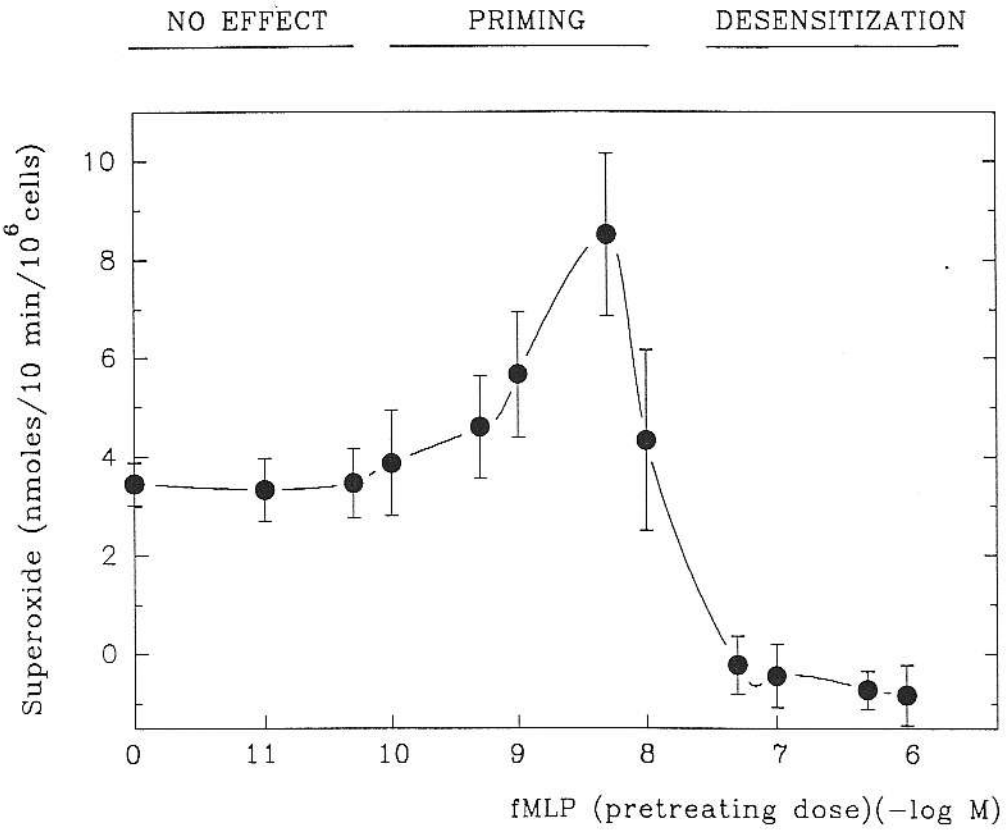

Figure 4 - Effect of pretreatment with increasing fMLP doses on O_2^- production by neutrophils stimulated with an optimal dose of fMLP. The response to a second fMLP addition ($5x10^{-7}$ M), depending on the fMLP concentration of the first addition (i.e., the pretreatment which causes homologous priming and desensitization) is shown. Neutrophils were incubated as described in Methods, and the incubation from the first to the second fMLP addition was carried out for 30 min. The O_2^- produced in the 10 min following the second fMLP addition is reported as mean value ± SD for triplicate assays.

the lack of, or the decrease in responsiveness may be induced by pretreatment of the cells with the same agent used for activation and is probably due to receptor down-regulation or desensitization[19-21].

To our surprise, we observed that pretreatment of neutrophils with a certain range of fMLP concentrations did not desensitize the cells, but primed them for a higher fMLP-dependent O_2^- production (Fig. 3). We called this phenomenon *homologous priming* and we carried out a number of additional experiments in order to characterize it[22]. A typical dose-response curve of homologous priming, representative of a large series conducted, is shown in Fig. 4. Homolo-

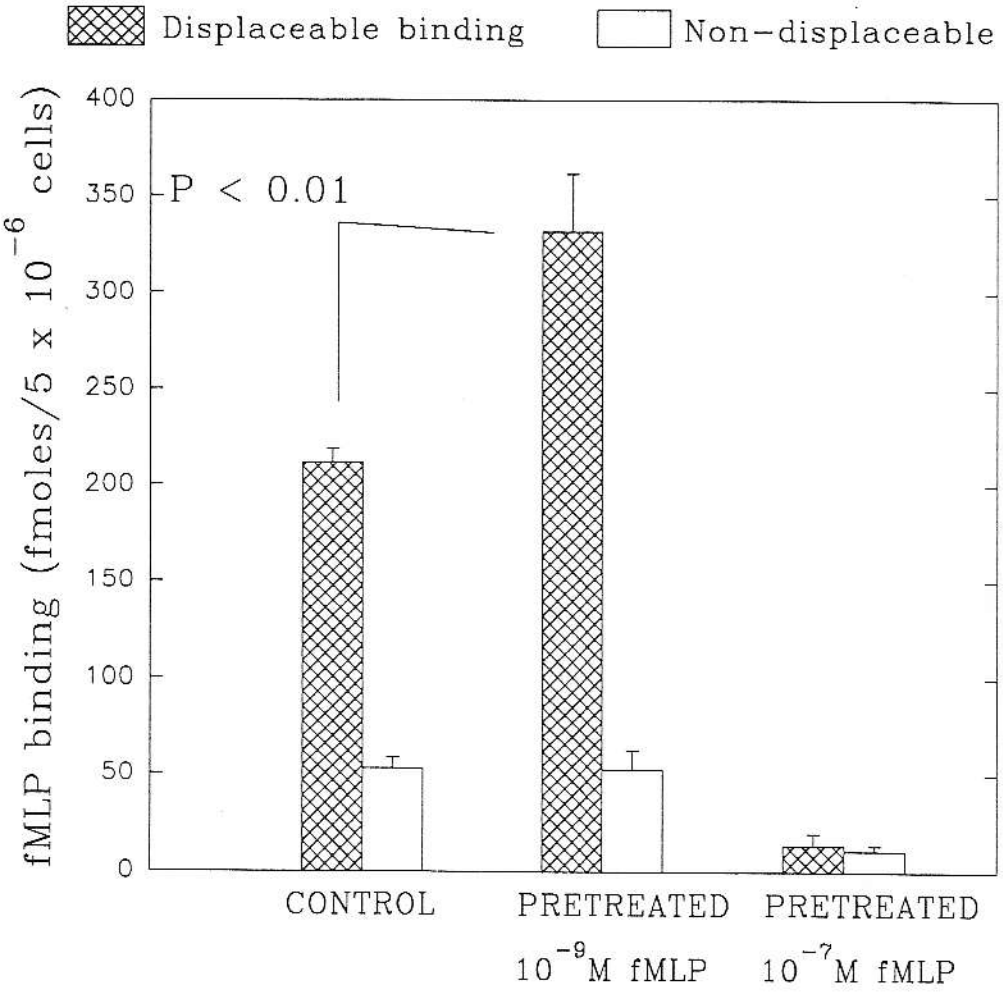

Figure 5 - Specific binding of 3H-fMLP to human neutrophils after the treatment with various doses of fMLP. Neutrophils were incubated for 15 minutes with the indicated doses of fMLP, and then rapidly cooled and centrifuged. The cells were suspended in ice-cold H-GCMA and the specific fMLP binding was determined as described in Methods. Values are mean ± SD for triplicate assays.

gous priming was caused by pretreatment with doses from 10^{-10} M to 10^{-8} M, while homologous desensitization was caused by doses of 10^{-8} M and upwards. Therefore, in these experimental conditions, the dose-dependence curve is biphasic in the sense that low doses appear to be stimulatory (priming), while high doses appear to be inhibitory (desensitization). These experiments are highly reproducible, provided the cells are kept in sterile, apyrogenic solutions throughout all the experimental manipulations.

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In parallel experiments, we found that cells exposed to priming doses of fMLP increased their fMLP membrane receptors, while cells exposed to high (stimulating) doses of fMLP dramatically reduced their fMLP binding capacity (Fig. 5)[22].

DISCUSSION

The experiments reported here show that the function of human neutrophils, assayed as oxidative metabolism activation and increase in adhesive properties, may be regulated in a number of ways by *in vitro* experimental manipulations.

These studies have provided two examples of inverse effects caused by the same compound at different doses. On LPS-treated cells, fMLP inhibits adhesion at low doses, while stimulating it at high doses; on normal cells, fMLP enhances subsequent activation of oxidative metabolism at low doses (priming), while inhibiting such metabolism when given at high doses (desensitization).

These apparently paradoxical results may be discussed in relation to two main aspects, i.e. *a*) the possible physiological meaning of such complex regulation of neutrophil behaviour and *b*) the contribution that make to our understanding of homoeopathic principles.

a) Homologous priming may be of pathophysiological significance, in the light of the complex role played by neutrophil free radicals and neutrophil enzymes in the host defenses and in the inflammatory processes. Formylated peptides and endotoxins, like the agents we used in our investigations, are major bacterial products[23] and can also be found in necrotic tissue[24] (probably due to protein fragments of mitochondrial origin). Therefore, significant amounts of these compounds may be released into the bloodstream and may prime circulating neutrophils in a number of conditions. One might expect that when phagocytes accumulate in the inflammatory focus and come into contact again with high doses of the stimulant, this may lead to an enhanced respiratory burst and degranulation.

Increased release of free radicals and enzymes is beneficial because it increases killing efficiency[25, 26], but can also have pathological implications with regard to tissue damage[27, 28]. Of course, our models represent an oversimplification of *in vivo* events, and we should also take into account the intervention of other regulating agents and the role played by desensitization when the cells are repeatedly exposed to high agonist concentrations.

The inhibition of adhesion by low doses of fMLP may also reflect the actual complexity of the regulation of neutrophil kinetics and functions. Both adhesion and detachment are necessary during directional migration: in this process, neutrophils take on a bipolar shape; adhesion occurs in the pole of the cell oriented towards the higher concentration of chemotactic agent, whereas in the rear

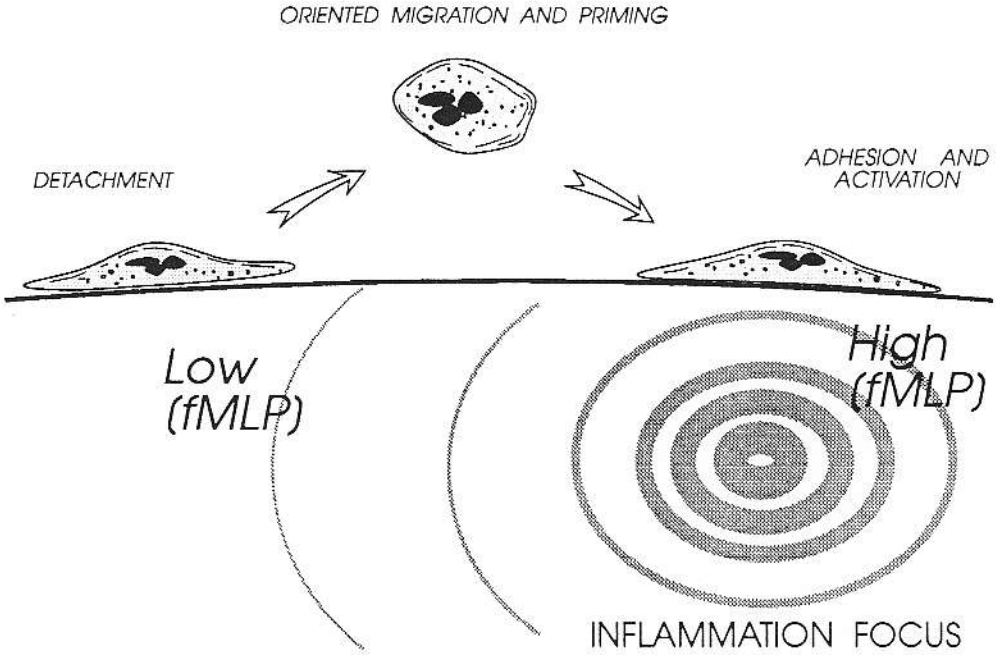

Figure 6 - An interpretation of the possible physiological significance of the inverse effects of low and high doses of fMLP on neutrophil adhesion. For details see text.

pole of the cells the membrane undergoes detachment from the adhesion surface. Adhesion and detachment are also important for the kinetics and distribution of leukocytes in the bone marrow, in the bloodstream and in the various districts where phagocytes are recruited to accomplish their defensive or destructive functions. Therefore, the regulation of adhesion/detachment processes is essential for the oriented migration of these cells.

It is known that a substantial proportion of neutrophils in the bloodstream are weakly adherent to endothelia and these represent the so-called marginated pool. Presence of endotoxins in the cell environment could enhance this phenomenon, leading to an increased proportion of adherent cells out of total circulating cells.

On this basis, it is possible to postulate (Fig. 6) that adherent cells on the outskirts of an inflammatory focus are affected by low doses of chemoattractants. These low doses induce the cells to detach and therefore to migrate towards the center of the inflammatory focus. At the same time, low doses of chemoattractants are capable of increasing receptor exposure on the cell surface and of priming neutrophils towards an enhanced response to subsequent challenge with homologous or heterologous stimulants. When the cells arrive at sites where the concentration of the agonist is higher, they re-express membrane anchor-

ing proteins and adhere to endothelia and other connective tissue structures. Expression of anchoring proteins may also help in the recognition and phagocytosis of bacterial targets or other foreign cells presenting suitable adhesion molecules. This model is consistent with the fact that chemotactic doses of fMLP are as low as the doses that cause detachment in our model system.

According to our data, the detachment does not reflect deactivation or exhaustion of neutrophils, but, on the contrary, represents an active response of previously adherent cells to low concentrations of chemoattractants. .

b) As far as homoeopathic issues are concerned, we have shown that Hahnemann's «similia principle» may be investigated from a scientific standpoint in a cell model. Many data have already been published in suppor of this suggestion.

For example, we need only to mention the studies showing anti-inflammatory effects of homoeopathic preparations of apis and histamine, two remedies which cause inflammation when given at high doses[29, 30] and the studies showing protective effects of homoeopathic preparations of toxic substances against the intoxication caused by high doses of the same compound[31, 32]. Inverse effects have been recognized for a long time in biology and pharmacology, where the terms «hormesis» or «Arndt-Schulz» law have been used to indicate the stimulatory effect of low doses of inhibitors or low doses of radiations[6, 7, 33-35].

Animal experimental models have clearly shown that the first interleukin discovered (IL-1), which has been implicated in the pathogenesis of arthritis, may reduce the severity and duration of antigen-induced arthritis when given as repetitive intraarticular injections[36]. Others have shown that oral administration of myelin may reduce the severity of experimental encephalomyelitis induced by injection of myelin[37]. In general, specific immunosuppressive therapy is a rapidly developing approach based on the fine and complex homeostatic regulation of helper and suppressor immunity pathways[38].

Therefore, the concept is emerging that Hahnemann's old aphorism «*similia similibus curentur*» may be rephrased in more modern terms as «*biologically active compounds may cause inverse effects on a cybernetically regulated, complex system when either the doses of the compound and/or the responsiveness of the target system are varied*».

These concepts may be regarded as an application of an analogic approach to scientific problems: the study of a simpler system (in this case, the white cell) may provide new insights into systems with higher degrees of complexity (in this case, the inflammation process in the whole organism). It has recently been pointed out that circulating blood phagocytes reflect the state of the immune response of the whole body[39]. We consider cells treated with bacterial toxins as a simplified model of cells from subjects affected by acute infections, who may have endotoxin in their bloodstream. Therefore, LPS-treated cells may be

considered as «diseased» cells, as compared with untreated cells, which may be considered as «healthy» cells.

Of course, clinical observations and experiments may be more directly relevant to homeopathy, but laboratory models have the advantage of greater simplicity and reproducibility.

In vivo studies of experimental inflammation[18, 40, 41] clearly indicate that neutrophils are endowed with highly sophisticated regulatory mechanisms, which appear to be stimulus-specific, depending on the type of agonists, on their doses and on the cell microenvironment. We have recently shown that granulocytes isolated from experimentally induced inflammatory exudate (skin window technique) are primed to some agents (fMLP, substance P) and desensitized to others (tumor necrosis factor)[18]. Others have reported that human neutrophils rapidly lose their TNF receptors after incubation with a variety of agents capable of stimulating their migratory and secretory responses[42].

All these results suggest that a change in the sensitivity and/or responsiveness to selected agonists may be one of the most important factors by which the release of toxic oxygen derivatives by neutrophils is controlled in the context of the inflammatory focus. A pattern of variable sensitivities is induced by the «disease» process (in our case, by the formation of an inflammatory focus).

These concepts are of obvious interest for the interpretation of inverse effects in clinical practice, because they demonstrate that the responses of a diseased — or otherwise stressed — biological system to regulatory molecules are different from the responses of a healthy system. This conclusion appears to be sufficiently supported by evidence obtained in a number of biological systems[43].

The scientific validation of the similia principle is within the reach of modern biology and pathology, and this important goal may be distinguished from the problem of the claimed effects of extreme homoeopathic dilutions. In other words, the «similia principle» may be investigated and interpreted independently of the so called «high dilution» or «high potency» effects. In fact, the «high dilution effect» is mainly related to the *possible mechanisms of information transfer* in physical and biological systems, while the «law of similarity» is mainly related to the *general rules of cybernetic regulation* of complex homeostatic systems. The study of this regulation may be important both at high and low dilutions or doses, according to the sensitivity of the system considered.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Consiglio Nazionale delle Ricerche. S.C. is the recipient of a research fellowship kindly provided by Guna s.r.l., Milano.

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