Ultra Low Doses

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SIMULTANEOUS MEASUREMENT OF OXIDATIVE METABOLISM ADHESION OF HUMAN NEUTROPHILS AND EVALUATION OF MULTIPLE DOSES OF AGONISTS AND INHIBITORS

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A testing model for the detection and evaluation of multiple doses of neutrophil agonists and antagonists is described. Incubations are performed in microtiter plates, where oxidative metabolism is measured as superoxide anion production and in the same assay system the adhesion is measured as activity of acid phosphatase. The test is simple to handle and is highly reproducible, and, therefore, it appears particularly useful for drug screening. Dose-response curves of various cell stimulants and inhibitors showed that the lowest effective doses ranged between 10-6 and 10-10 M according to the agents employed. Adhesion appeared to be more sensitive to low doses than superoxide production.

INTRODUCTION

Neutrophil granulocytes play a key role in the host defence against bacterial infections and also in other pathologic processes related to inflammatory reactions. When pathogenic agents invade a tissue, neutrophils are recruited from the bone marrow and the blood compartment into the inflammed area. At the inflammatory site, neutrophils and endothelial cells express membrane anchoring molecules and receptors that mediate the adhesion of the leukocytes to the vessel wall. Neutrophils then orientate and migrate through a gradient of chemotactic factors towards the

centre of the inflammation, where they interact with the etiopathogenic agent. At this stage, phagocytosis of foreign particles, release of lysosomal granule constituents and activation of the oxidative metabolism, with production of superoxide anion (O_2^-), hydrogen peroxide and other toxic oxygen derivatives rapidly occur.

All these events are regulated by a variety of mediators, some of which are of exogenous origin, like vegetable or bacterial compounds (e.g. phorbol esters, lectins, peptides, lipopolysaccharides, toxins, etc.), while others are produced endogenously, like complement fragments, kinins, cytokines, neuropeptides, growth factors, eicosanoids, platelet-activating factor, adenine nucleotides, etc. Most of these factors act on specific membrane receptors and may affect neutrophil functions essentially in three ways : 1) by directly triggering one or more cell responses such as adhesion, chemotaxis, phagocytosis, respiratory burst, secretion of lysosomal constituents, b) by priming the neutrophils, that is by including in the cell a state of enhanced responsiveness to a subsequent stimulus, c) by inhibiting, or de-sensitizing, the neutrophil, whose functions may result dampened. Activation, priming and inhibition are subtly controlled by physiological mechanisms and could be the target, at least in theory, of pharmacological manipulation. Priming and activation are required and desirable during microbial infections and should be exogenously reinforced particularly in immunocompromised hosts, while inhibition and de-sensitization may be necessary in order to prevent the toxic and damaging effects of neutrophils during immunopathologic processes, intravascular aggregation, post-ischemic tissue injury, etc. [1-3].

The mechanisms of action of various compounds active on neutrophils may be better investigated using methods that evaluate more than one function. In this report we describe a novel microplate assay system that allows the simultaneous evaluation of the oxidative metabolism (measured as superoxide anion release) and of the adhesion of neutrophils exposed to a variety of agents. Several classic leukocyte stimulants were tested : the bacterial peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), the lectin Concanavalin A (Con A), the active principle of Croton oil phorbol-12-myristate-13- acetate (PMA), and yeast particles in the form of serum-treated zymosan (STZ). We also tested the effects of endotoxin, the

lipopolysaccharide (LPS) component of the cell wall of Gram negative bacteria, a compound that has been previously shown to act as a powerful priming agent [4,5]. Finally, we report data of experiment where the cells have been treated with inhibitors before stimulation. The inhibitors utilized in this study were N-ethyl-maleimide (NEM), a toxic and irritating compound that strongly interacts with functional sulfhydryl groups [6], adenosine, a molecule with many physiological roles including effects on leukocytes [7], and corticosteroids, that are largely used as anti-inflammatory agents and that in previous papers have been shown to inhibit the leukocyte metabolism [8,9].

METHODS

Materials

fMLP zymosan, PMA, Triton X-100, NEM, LPS (from E. Coli, serotype n. 026.B6) were purchased from Sigma Chem. Co.,St-Louis, M.O. Cytochrome c and adenosine were from Boehringer, Mannheim, F.R.G.. Concanavalin A was Vector Lab. Inc., Burlingame, CA. Human copper-zinc superoxide dismutase (SOD) was a gift of Prof. J.V. Bannister (Cranfield Institute of Technology, Bedford, U.K.). Fetal bovine serum (FBS) was from Flow Laboratories. Serum was inactivated by incubation at 56°C for 1 h. Percoll was from Pharmacia, Uppsala. Sterile 96-well microtiter plates with flat bottom wells were of type Linbro, from Flow Laboratories. Hank's balanced salt solution (without calcium and magnesium)(HBSS) was from Gibco Ltd, Paisley, Scotland. Other materials and reagents were of the highest purity available. In order to avoid any contamination that could cause artifactual activation or priming of the cells, sterile solutions and disposible plasticware were used throughout all the experimental procedures, that were carried out, when possible, under a laminar flow hood. Reagents were prepared using clinical apyrogen water or 0.9% NaCl solutions.

Human neutrophils were prepared from EDTA-anticoagulated blood by centrifugation over Percoll gradients. Percoll was diluted with distilled water and then with 1/10 volume of 10 x phosphate-buffered solution (PBS) in order to have the desired concentration in1 x PBS (KH2PO4 0.144 g/liter, NaCl 9 g/liter, Na2HPO4 0.795 g/liter, pH 7.4). Starting from 40 ml of blood, two 50 ml sterile plastic tubes were layered (from bottom) with : 15 ml of 73% Percoll, 15 ml of 62% Percoll, 20 ml of blood. The tubes were then centrifuged for 20 min at 1800 RPM

with a Sorvall T6000B centrifuge. Neutrophils were recovered as a broad band at the 73%-62% interfacie, were diluted with 1 volume of PBS, and centrifuged for 10 min at 1200 RPM. The pellet was usually slightly contaminated by erythrocytes, that were lysed by a brief hypotonic shock : cells were suspended in 20 ml of 0.2% NaCl for 20 seconds, then the isotonicity was restored by addition of 20 ml of 1.6% NaCl and right pH was restored by addition of 10 ml of PBS. The cells were then centrifuged for 10 min at 1200 RPM, washed once with 50 ml of PBS, then finally suspended in HBSS supplemented with 5 mM glucose, 0.5 mM Cacl2, 1 mM MgSO4, 0.2% human serum albumin (solution H-GCMA). Cells were counted and the concentration was usually brought to 2.7 x 10^6/ml with the same suspension buffer. the yield ranged from 6 to 11 x 10^7 cells, and more than 98% of purified cells were neutrophils.

Dilutions of stimulants and inhibitors

Stock solutions of compounds used in this work were prepared and stored as follows : fMLP (10-4M) and PMA (10-4M) were dissolved in dimethylsulfoxide and stored at -70°C. Zymposan was suspended in HBSS supplemented with 0.5 mM CaCl2 and 1 mM MgCl2, was opsonized with 50% pooled human sera for 30 min at 37°C, was centrifuged and washed with the same buffer, then was suspended in 0.9% NaCl at the concentration of 20 mg/ml. Aliquots of this suspensions were frozen at -20°C and thawed the day of the experiment. Concanavalin A (10-4M, using the mol. weight of 104,000), NEM (3 x 10-2M), hydrocortisone (10-3M) were dissolved in 0.9% NaCl and used the day of the experiment. LPS (2 x 10-4M, using a mean mol. weight of 10,000) was dissolved in HBSS and stored at +4°C. Dexametasone (4 x 10-3M) was dissolved in ethanol and stored at +4°C.

When indicated, dilutions were done as follows : the stock solution was diluted with 0.9% NaCl to a concentration exactly 4 times higher than that required for the maximal dose to be employed in the planned experiment. This has been done because each agent becomes diluted 4 times in the microwell incubation mixture. This maximal dose was then used for preparing a further series of either 2x or 10x dilutions until the lowest dose required. for dilutions, 16 x 100 mm polyethylene disposable tubes were used. 2x dilutions were made by adding 0.5 ml of the more concentrated solution to 0.5 ml of 0.9% NaCl. 10x dilutions were made by adding 0.2 ml of the more concnetrated solution to 1.8 ml of 0.9 NaCl. Immediately after dilution, each solution was stirred

for 20 seconds with a Vortex tube mixer. Diluted solutions were used only the day of the experiment.

Coating the microplates

Preliminary experiments showed that neutrophils incubated in microplates spontaneously adhere to the bottom of the well in less than 30 min and produce considerable amounts of superoxide. This prevents the possibility to study the cell adhesion and metabolism upon addition of specific stimuli. Nonspecific activation was totally abolished by coating the microplate wells with serum proteins. In order to use safe, sterile and standardized serum preparations, fetal bovine serum (FBS) was used for this purpose. 100 µl of 50% FBS (diluted with PBS) were delivered in each well and the plate was incubated for at least 1 h at room temperature. Immediately before use, the plates were washed three times with PBS by using an automatic plate washer (Easy Washer 2, SLT Labs Instruments).

Assay of superoxide production and of adhesion

In synthesis, superoxide anion was measured by the SOD-inhibitable reduction of ferricytochrome c. At the end of the incubation, the assay mixture and unhadherent cells were washed out, while remaining adherent cells were quantitated by enzymatic assay.

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, etc.), on the number of compounds and of controls to be tested. After preliminary experiments, when multiple concentrations of stimulants or of inhibitors were tested, the scheme that was adopted is the following : Rows B, D, F, contained 25 µl of test compounds. Therefore 12 different doses in triplicate were tested for each plate. Rows C, E, G, contained 25 µl of 0.9% NaCl, or of dilutions of the solvent used in the preparation of the test compound. In row H, four wells were used as blank without cells (H9-H12), and in the other wells a known number of cells in a small volume (5-10 µl containing 2-20 x 104 neutrophils) was added after washing. These cells were used as standard for the calculation of the % of adhesion. Row A was used when other controls were required, i.e. assays in the presence of SOD (25 µl of 0.9% NaCl with or whitout test compound containing 0.5 mg/ml SOD) or blanks of the test compound plus cytochrome c (to check for possible nonspecific reduction of cytochrome c by test compound). These control experiments demonstrated that in all the

experimental conditions employed, the inclusion of SOD totally inhibited cytochrome c reduction, while it did not affect the adhesion of neutrophils. Therefore, SOD was not currently included as control in all dilutions of agonists and of inhibitors, and the reduction of cytochrome c was taken as the measure of superoxide production. This greatly increased the versatility of the test without affecting its precision.

The plate was brought to 37°C, then 75 µl of pre-warmed neutrophil suspension were added to each well, except blanks. By using automatic pipette, this operation required about 60-90 seconds. Usually 2 x 105 cells/well were added, because control experiments demonstrated that this is the optimal cell number to have good sensitivity in the range of cell concentrations where both superoxide and adhesion were linear (between 5 x 104 and 3 x 105 cells/well). At this point two different schemes were used, according to the object of the experiment : scheme 1. In experiments where multiple stimulants or multiple doses of stimulants were tested, the reaction was started by addition of neutrophils plus cytochrome c. Just before delivering to the plate, the cell suspension was supplemented with a little volume of concentrated solution of ferricytochrome c, in order to obtain a final concentration of 0.15 mM. The plate was then incubated at 37°C in cell culture incubator (5% CO2) for the desired time, then measured as detailed below. Scheme 2. In experiments where priming agents or inhibitors were tested, the neutrophils were dispensed in the plate without cytochrome c, then incubated for the indicated period of time at 37°C. After this pre-incubation, the wells were rapidly supplemented with 50 µl of a solution H-GCMA that was pre-warmed at 37°C and contained 0.45 mM ferricytochrome c (final concentration : 0.15 mM) plus the stimulatory agent 3x concentrated with respect to the final concentration in the assay mixture. The reaction was then incubated as describedin scheme 1. In all the procedures, care was taken to avoid cooling of the plate when it was taken from the incubator for filling or reading.

At the end of the incubation, the plates were rapidly transferred into a microplate reader (Reader 400, SLT Labs Instruments) and the reduction of cytochrome c was measured by reading the plates at 550 nm using 540 nm as reference wavelenght to avoid interferences due to light scattering. Quantitation of absorbance of standard amounts of reduced and oxidized cytochrome c established that in this system the reading of 1 nmole of reduced minus oxidized

for 20 seconds with a Vortex tube mixer. Diluted solutions were used only the day of the experiment.

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cytochrome c (that is 1 nmole of 02-) is 0.037 O.D. Reading the plate took about 10 seconds.

Immediately after reading cytochrome c reduction, the plate was transferred to the automatic washer and subjected to two cycles of washing with PBS at room temperature. Washing was carefully calibrated because this was essential in order to obtain an optimal sensitivity and reproducibility of adhesion measurements. Each cycle was done as follows : emptying by rapid aspiration, filling by slight jet for 1.5 seconds, soaking for about 30 seconds, emptying by aspiration. The overall washing procedure took about 90 seconds. Adherent cells were quantitated by measuring the membrane enzyme acid phosphatase. 75 µl of 0.1 M acetate buffer, pH 5.3, containing 0.2% Triton X-100 were dispensed into the wells. After 5 min at room temperature 75 µl of the 0.1 M acetate buffer, pH 5.3 containing the substrate 10 mM p-nitrophenyl-phosphate were added. The reaction was incubated at room temperature for 20 min., then it was blocked by addition of 100 µl of 2 N NaOH. The p-nitrophenol produced by the phosphatase reactions was measured with the microplate reader at 405 nm. The % of adherent cells was calculated on the basis of a standard curve obtained with a known number of cells.

RESULTS

Optimal experimental conditions and kinetics

The first series of experiments was done in order to establish the optimal conditions for exploring metabolic and adhesion functions of neutrophils in our assay system. We found that the reduction of cytochrome c (measurement of superoxide production) and the acid phosphatse reaction (measurement of adhesion) are linear in a range of cell concentrations between about 5 x 104 and 3 X 105 neutrophils/well. The subsequent experiments were therefore performed by using 2 x 105 cells/well. This sensitivity allowed to carry out experiments on two-three plates starting from 40-ml blood samples.

Studies on low and ultra-low doses require high precision and accuracy of the test.

Table 1. Precision of microplate assay for neutrophil adhesion and superoxide production. Pre-warmed cells added to several random wells of the microplate containing stimulants or 0.9% NaCl (see scheme 1 of the methods). Zymposan : 0.4 mg/ml ; fMLP: 10-7 M. 02- : nmoles/106 neutrophils ; adhesion : % of total.

Test	Stimulant		Results		
			Mean	S.D.	C.V.
24 identical wells	None (0.9 % NaCl)	O_2^-	0.8	0.3	37.5
		ADHESION	1.7	0.4	23.5
48 identical wells	Opsonized zymosan (60 min)	O_2^-	11.5	0.9	7.8
		ADHESION	48.9	2.8	5.7
36 identical wells	fMLP (30 min)	O_2^-	7.6	1.1	14.4
		ADHESION	25.6	3.2	12.5

Table 1 reports data on superoxide production and adhesion of multiple replicate assays carried out in random wells of the same plate. It can be seen that in the absence of stimulants the spontaneous activation is extremely low. This is particularly important when very low doses of stimulants and bordeline responses have to be evaluated. When the cells were challenged with either a phagocytosable agent (opsonized zymosan) or a soluble stimulant (the bacterial tripeptide fMLP), both superoxide anion production and the adhesion were markedly stimulated. Comparing the stimulated with the resting cells demonstrates that the assay is very sensitive in order to reveal specific activation. Standard deviations and variation coefficients were very low, demonstrating that the test is sufficiently precise and reliable. Comparing the results obtained with stimulated cells by using different blood samples in different days showed that inter-individuals variations are in the range of the 17-63% for superoxide production and in the range of 14-39% for adhesion according to the different stimulants used (data not shown). Therfore the test is more suitable for multiple tests on the same cell preparation than when used for studies on the responses of different subjects.

Figure 1 shows the time-course of the superoxide production and the contemparaneous adhesion of neutroplis. All the stimulants employed induced both responses in neutrophils, but differences were noted in the kinetics of the two phenomena. With PMA as stimulant, adhesion was quite precocious with respect to metabolic activation, but at 20 min the adhesion reached a plateau, then slightly declined.

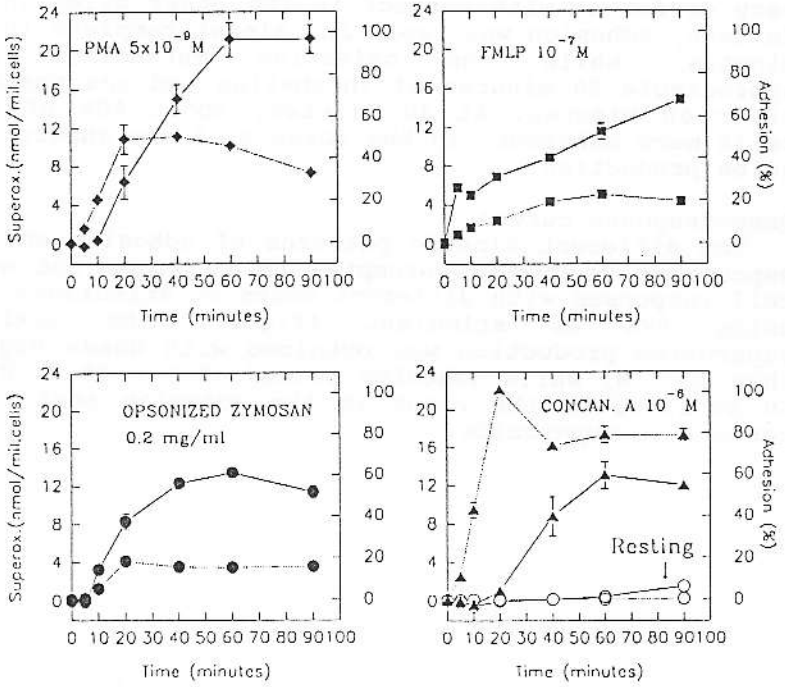


Fig.1. Time-course of superoxide production and adhesion of neutrophils in response to various stimulants. Solid line : superoxide ; dotted line ; adhesion. Assays carried out according to scheme 1 described in Methods.

The superoxide production with PMA was linear until 60 min. With fMLP, a very rapid initial burst of superoxide production occured in the first five minutes, then oxidative metabolism slowed down and continued linearly. The corresponding adhesion was very slow, reaching a maximum of 20% adherent cells 60 min from the stimulation. Opsonized zymosan induced, after a 5 minutes lag, sustained metabolic response, while in the same conditions the adherence was low and completed in 20 min. In the presence of the lectin Concanavalin A neutrophils behaviour was very different with respect to the other stimulants. In fact, adhesion was rapid and almost complete in 20 minutes, while the oxidative metabolism was appreciable 20 minutes of incubation and was maximum after 60 minutes. At 10 minutes, about 40% of the cells were adherent, in the absence of any superoxide anion production.

Dose-response curves

The different kinetic patterns of adhesion and of superoxide production prompted us to study the same cell responses with different doses of stimulants. By using PMA as stimulant (fig.2), the maximum superoxide production was obtained with doses higher than 10-7 M, while adhesion peaked at 5 x 10-9. E.D. 50 schifted to the right in the adhesion test with respect to superoxide.

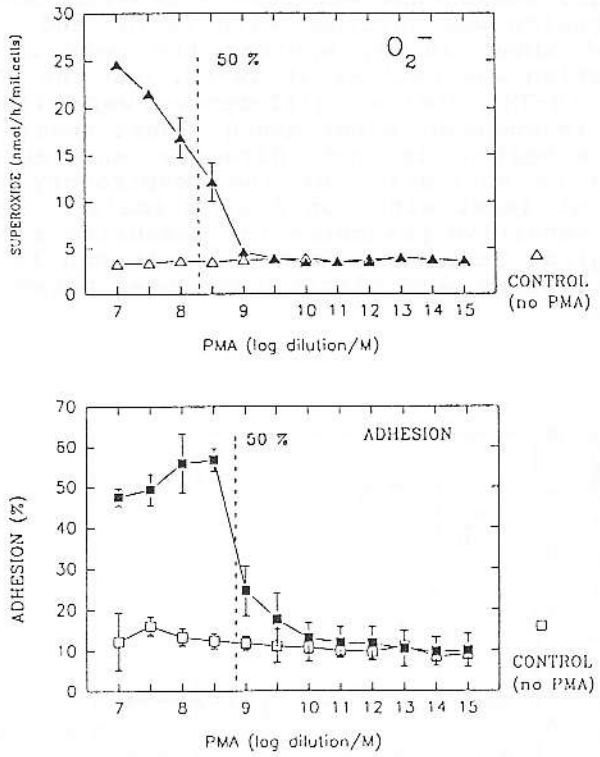


Fig.2. Activation of neutrophils by decreasing doses of PMA. In this experiment, cells were pretreated with 2 x 10-8 M, fMLP, for 5 min, then stimulated with PMA for 1 h.

Fig.3 shows that with Con A as stimulant the peak of adhesion was obtained with 10-7M, and the E.D. 50 was of about 10-8M, whereas the peak of metabolic activation was optimal at 10-6M, and the ED50 was of about 10-7M. Similar differences were found in two other independent experiments. These results indicate that adhesion is not directly and automatically linked to activation of the respiratory burst, and that, at least with Con A as stimulant, adhesion is amore sensitive parameter for measuring a neutrolphil biological response. From fig.3 it can be seen that no activities were elicited by doses between 10-9 and 10-20M.

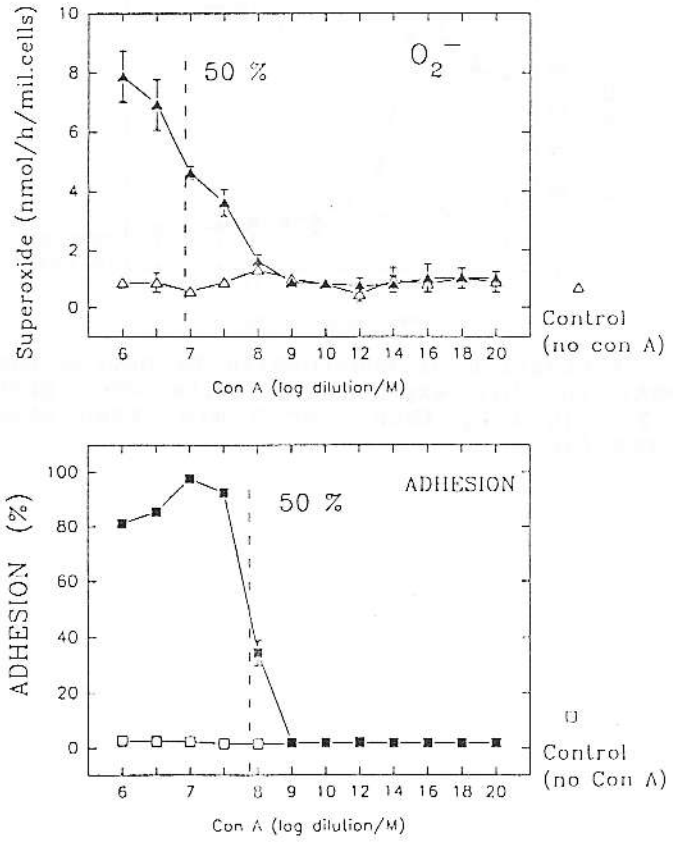


Fig.3. Activation of neutrophils by decreasing doses of Con A. Incubation time : 60 minutes.

Fig.4 shows the dose-response of the stimulation induced by fMLP. In this case the peak(10-7M) and the ED50 (about 4 x 10-8M) were similar for both superoxide production and adhesion. Careful inspection of the results of adhesion showed that a small percentage of cells were adherent even with 10-9M fMLP. The difference between data with 10-9M fMLP and control matched undstimulated cells was significant with $p<0.05$ (paired student t test). A similar small peak in this "ultra low dose" range was obtained in another experiment, but it was not reproduced in a further one. Figure 4 also reports the results of parallel assays carried out using cells that had been previously treated with a low dose of fMLP. This treatment caused the cells to exhibit a slightly higher basal superoxide production, but their oxidative metabolism was totally unaffected by a subsequent stimulation by any dose of fMLP. This is probably due to receptor desensitization [10].

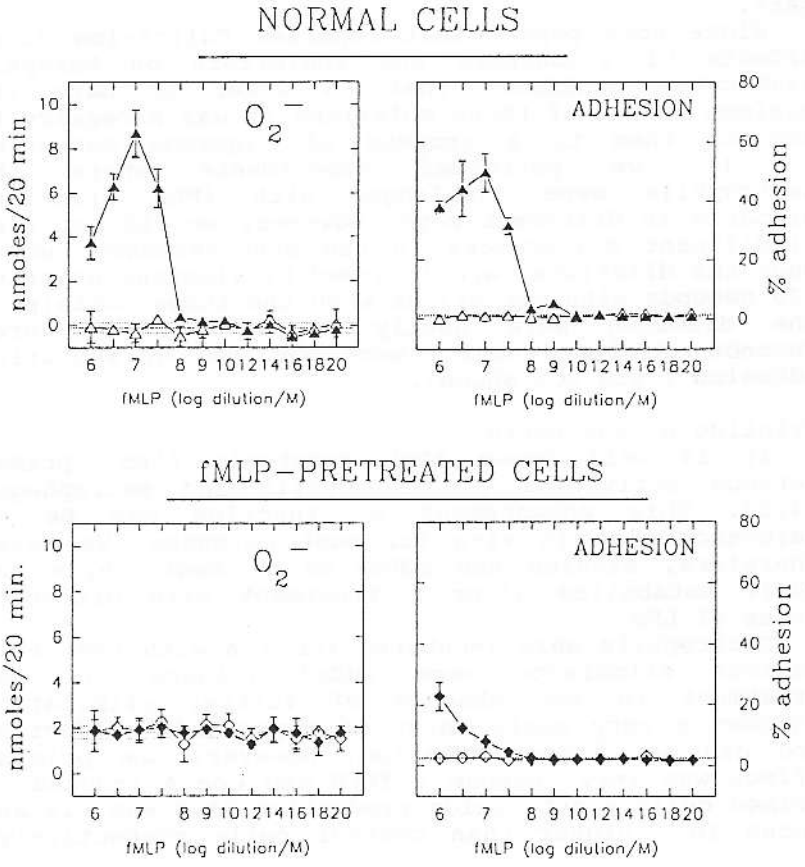


Fig.4. Activation of neutrophils by decreasing doses of fMLP. Normal neutrophils and neutrophils pre-treated for 10 min with 2 x 10-8M fMLP were added to microwells containing the indicated doses of fMLP and the superoxide production and adhesion were measured as described in methods (scheme 1). Hollow symbols are control cels, unstimulated, with 99% confidence limits (dotted lines).

Also the capability to adhere to surfaces was desensitized, although not to the same extent as metabolic activity. In fact, with 10-6M fMLP as a second stimulus, at least 20% of the cells were adherent. Therefore, in this experiment a condition of activation of adhesion without activation of superoxide release
is described.

This further indicates that the two events are possibly controlled by mechanisms that are different at least in
part.

Since some papers that reported "ultra-low dose" effects of stimulants and inhibitors on basophil leukocytes mentioned that in order to have the maximum effect of these solutions it was necessary to subject them to a process of vigorous succusion [11-13], we performed experiments where the neutrophils were challenged with fMLP dilutions prepared in different ways. However, we did not find significant differences in the dose-response curves when the dilutions were followed by vigorous stirring (20 seconds with Vortex) or when the tubes containing the dilution were gently tapped for two-three seconds. Similar results were obtained by measuring adhesion (data not shown).

Printing by endotoxin

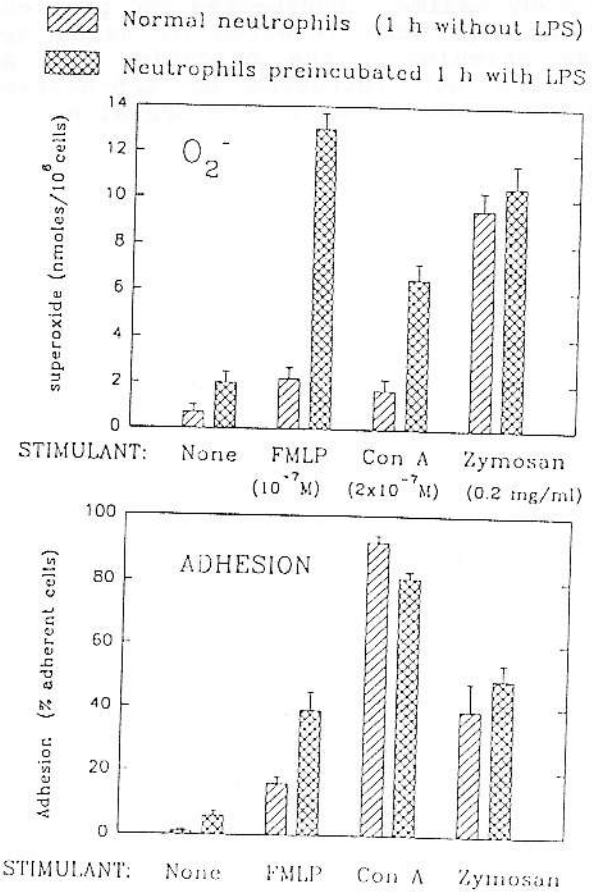
It is well known that endotoxin (LPS) primes various activities of neutrophils and macrophages [4,5]. This enhancement of function may be of relevance also in vivo for host defenses. We have, therefore, studied the adhesion of neutrophils and their metabolism after a treatment with different doses of LPS.

Neutrophils were incubated for 1 h with LPS, then various stimulants were added (figure 5). LPS treatment in the absence of further stimulation induced a very small burst of superoxide production and did not induce adhesion. However, the priming effect was very intense : fMLP and Con A induced in primed cells a superoxide production that was six and three fold higher than control cells respectively.

The adhesion response to fMLP was also primed by LPS, although to a lesser degree than O2-production. Con A-induced adhesion was not primed, probably because it was already maximal. Unespected and previously not reported findings were revealed by using opsonized zymosan as stimulus : the oxidative and adhesion functions were not increased by LPS-pretreatment, excluding the small effect of the LPS alone.

b

Fig. 5. Priming of neutrophil responses by LPS (10-8M, 1 h). After stimulation, cells were incubated for 40 minutes.



This indicated that, at least in these experimental conditions, priming does not involve increase of the effector systems necessary for 02- or phagocytosis (e.g. NADPH oxidase and contractile apparatus respectively), but involves other regulatory steps that are probably more proximal to the receptor and membrane transduction systems.

The changes of cell activation apparatus that are responsible for priming by LPS are under investigation, but it is conceivable that these changes are more "subtle" and easy to be triggered by low doses. Therefore we looked for possible low-dose and ultra-low dose effects of priming by LPS. The data of figure 6 show that LPS pretreatment primed the cells to respond to fMLP at doses as low as 10-8M, but the effect decreased rapidly at further dilutions and became undetectable below 10-10M. The adhesion appeared to be slightly more sensitive to priming than the superoxide production. In any case, since 10-8M corresponds to approximately 0.1 μg/ml, it can be speculated that in 5 liters of blood a similar effect on circulating neutrophils could occur in vivo by a dose as low as 0.5 mg of endotoxin.

Effects of inhibitors

The assay described in this paper could be very useful for screening of drug effects on two peculiar leukocyte functions. Besides the inhibitory agents used as tools for the study of neutrophil biochemistry, also the drugs used in clinical as anti-inflammatory agents are currently characterized in vitro models. Here we report some experiments using three compounds that have been shown to have antagonistic activity in various functions of neutrophils (excluding adhesion, that has been studied very rarely). This approach was chosen in order to check the validity of the method for this kind of research and also to look for possible inhibition effects in the ultra-low dose range.

The sulfydryl reagent n-ethyl maleimide was found to be an extremely potent inhibitor of both the evaluated leukocyte functions, that were totally blocked at doses of 10-5M irrespective of the stimulatory agent utilized (fig.7). However, by examing the various dose-response curves, we noted some differences : First, the stimulation by fMLP was more sensitive to inhibition than the stimulation by zymosan. Second, with STZ as stimulus, the adhesion was blocked at doses of NEM (5 x 10-6M) that were much less inhibitory on the superoxide production. These data indicate that selected functions (adhesion) and selected receptor-specific responses (e.g. activation by fMLP) are more dependent on the integrity of functional sulfhydryl groups.

Fig.8 shows the effect of a different type of inhibitor, the nucleotide adenosine, that interacts with specific membrane receptors and has been shown to inhibit the superoxide anion production but not the aggregation of neutrophils [7,14-15]. It has been

suggested that adenosine exerts its inhibitory effects probably by increasing the intracellular levels of CAMP [16]. In our conditions, adenosine inhibited both superoxide generation and adhesion induced by fMLP, with similar dose-response curves.

Finally, we tested the effects of corticosteroids such as dexametasone and hydrocortisone at doses between 10-5M and 10-15M. Contrary to what reported by others [8,9] these agents were totally ineffective on the considered neutrophil functions, even at doses much higher than those, used in corticosteroid therapy (data not shown).

Fig.6. Dose-response of the priming effect of LPS. Neutrophils were pretreated for 1 h with various doses of LPS, then they were stimulated with 10-7M fMLP, according to the scheme 2 of the methods. Filled triangles : pretreatment with LPS, stimulation with fMLP ; open triangles : control cells pretreated with dilutions of dimethylsulfoxide in 0.9% NaCl, stimulation with fMLP ; closed squares : pretreatment with LPS, no stimulation.

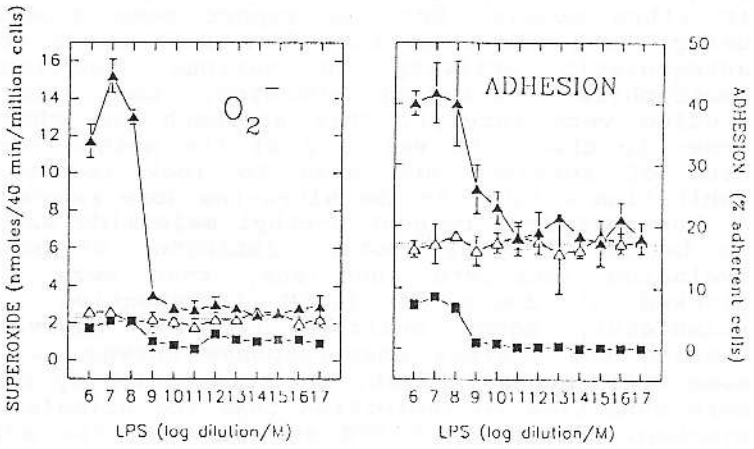
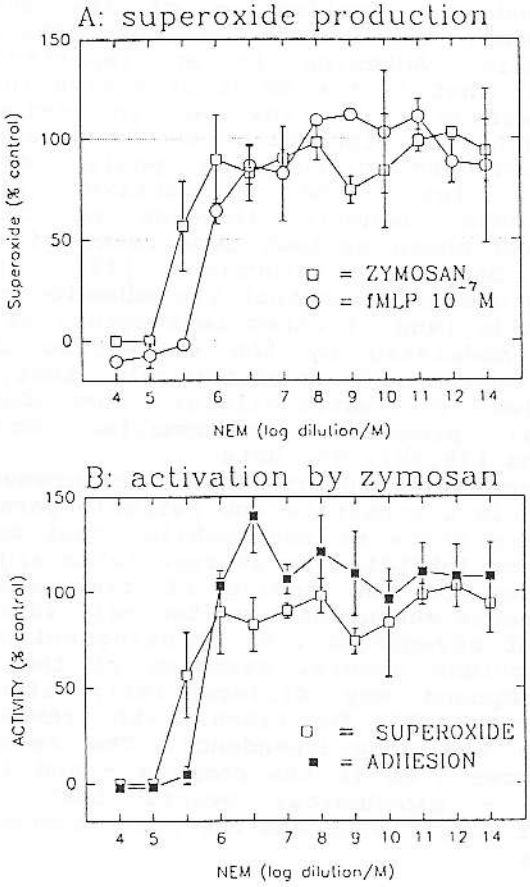


Fig.7. Effects of NEM on neutrophil functions. Cells were pretreated for 1 h with NEM, then stimulated with 10-7M fMLP or 0.2 mg/ml opsonized zymosan for 30 min.



DISCUSSION

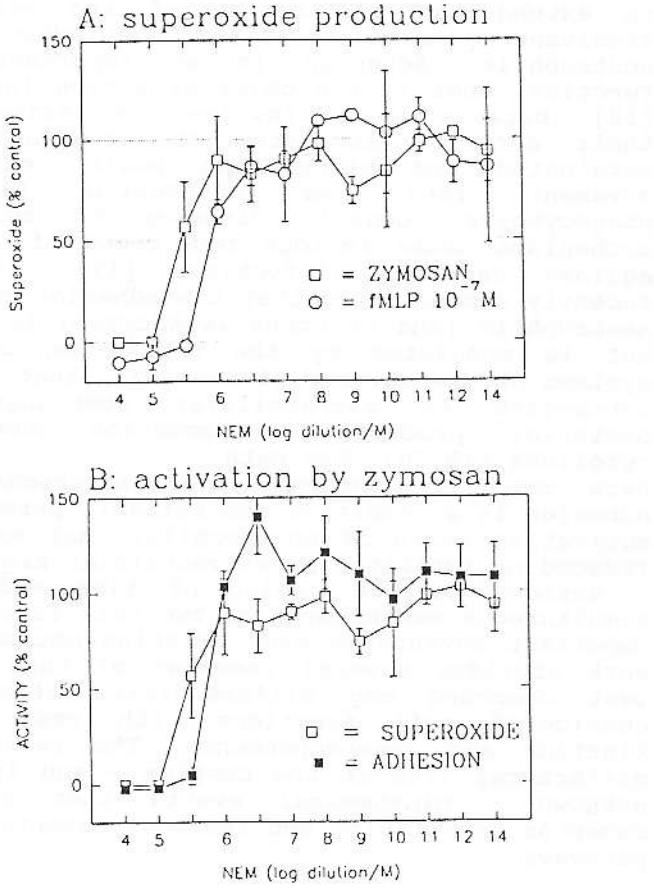
We have described a testing system for the detection and evaluation of stimulatory and inhibitory compounds of neutrophils. The assay is sensitive and versatile, allowing to perform a two-three plates experiment starting from a relatively small amount of blood (40 ml), that can be easily obtained as suplemental sample from blood donors. for each plate, up to 12 different doses of a test compound may be evaluated in triplicate with controls no blanks.

This test is based on a microplate assay for superoxide production by leukocytes, similar to others that have been previously reported [17]. We have established experimental conditions that allow to extend the application of the assay to the simultaneous measurement of the adhesion of the neutrophils. Adhesion is an important leukocyte function, that is the objet of active investigations [18], because it is involved in various steps of their activity including the regulation of the marginated and circulating pools, extravasation, movement into the connective tissue, and phagocytosis. Genetic diseases of cell adhesion mechanisms cause serious impairment of the defenses against bacterial infections [19]. It has been recently demonstrated that the adhesion capability of neutrophils (and of other leukocytes) is not stable, but is modulated by the expression of anchoring systems on the surface of the cell, that, in turn, is controlled by extracellular compounds such as bacterial products, inflammation mediators and cytokines [18,20]. The data here reported confirm that measurement of cell adhesion is a sensitive and reliable parameter of the activation state of neutrophils, that may be either induced or inhibited by extracellular signals.

Besides to the saving of time and materials, simultaneous measurement of two cell functions offers important advantages over existing methodology. This work provides several examples of the fact that a test compound may differentially affect the two considered cell functions with respect to both kinetics and dose-dependence. The reason of these differences lies in the complex - and in part still unknown - biochemical events that regulate the receptor sensitivity and the post-receptor signalling pathways.

Fig.8. effects of adenosine on neutrophil functions. Normal neutrophils and neutrophils that were pre-treated with 10-8M LPS were treated for 10 min with adenosine, then were stimulated with 10-7M fMLP for 20 min.

Fig.7. Effects of NEM on neutrophil functions. Cells were pretreated for 1 h with NEM, then stimulated with 10-7M fMLP or 0.2 mg/ml opsonized zymosan for 30 min.



DISCUSSION

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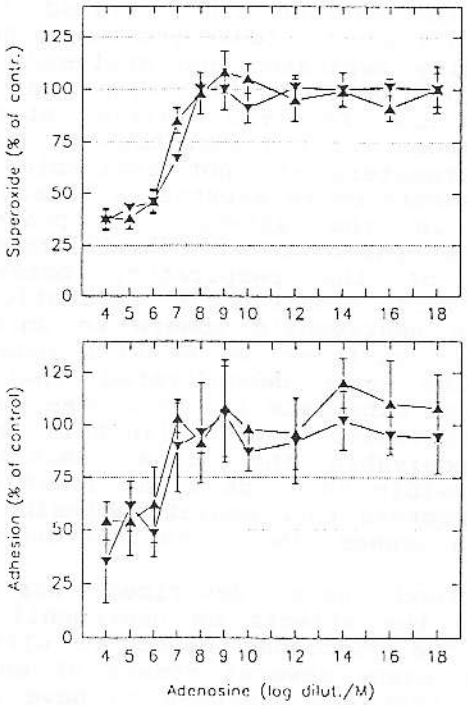

▼ = normal neutrophils
▲ = LPS-pretreated neutrophils

Analysis of each of the experimental findings here reported is outside the scope of this paper, that is methodologically-oriented. In general, the following indications emerge from our findings : a) stimuli that activate the oxidative metabolism also activate the adhesion, but the kinetics of the two phenomena vary according to the stimulus, b) the two considered responses may be differentially affected by agonists or inhibitors, c) adhesion may occur in the absence of superoxide production : low doses of stimulants such as Con A (fig.3) or high doses of fMLP in desensitized cells (fig.4) can induce adhesion without activation of the respiratory burst, d) adhesion is not a pre-requisite for metabolic activation and for the increased responses observed after priming by LPS.

The above conclusions are in agreement with the view that the various signal-transduction pathways for the different leukocyte responses may be experimentally separated and analysed. So far, the relationship between adhesion and metabolic activation has received little attention, also because methods for the simultaneous quantitation of the two parameters are not available. The classic view that adherence to substrates causes spreading of the cell in the attempt to phagocytose them ("frustrated phagocytosis") and therefore causes activation of the respiratory burst should be reviewed. This simultaneous activation undoubtely occurs when neutrophils adhere to uncoated plastic wells, or to surfaces coated with immuno-complexes, but we have here demonstrated that on a more physiological substrate (in this case serum proteins) and with particular agonists (in this case Con A, but it is conceivable that other lectins can cause similar possible that Con A, at least at low doses, binds toreceptors that mediate adhesion, but not the burst, in a manner similar to that described for C3b [21].

The method here described was adopted to investigate the effects on neutrophil functions of some selected compounds usqed at ultra-low doses, that is at doses several orders of magnitude lower than those that are expected to have any effect on the basis of previous knowledge. The rationale of this approach is based on the fact that recent papers reported that solutions of stimulants and inhibitors that had been prepared accoding to special dilution procedures (very close to those employed by homeopathic pharmacopea) exhibited a biological activity even in the ultra-low dose range [11-13, 22-24]. In particular, it was reported that in the first dilution steps the activity decreased, as expected, but further dilutions caused the re-appearance of the activity, that was therefore attributed to some unknown "meta-molecular" [13] biological effect. These paradoxical observations raised a number of questions and also controversies based on methodologic considerations [25-27]. However, in our opinion, these reports were of interest because pointed to the possible existence of previously unrecognized biophysiacl phenomena and because they documented the effort of setting-up laboratory models of investigation of ultra-low dose pharmacology that, as it is well known, has a wide application in clinical practise in a number of countries. We therefore did not consider

"unbelievable" [25] this approach and we tried to reproduce, using our model system, similar effects.

The experiments here reported show that we were not able of obtaining ultra-low dose effects on neutrophil superoxide production and adhesion. Active doses were very low, especially in the adhesion tests, but these doses probably can not be defined as ultra-low in the sense that they might exert their action through some meta-molecular effects. However, these are only preliminary studies, and it can not be excluded that by using different preparations of test compounds or a different experimental design, such effects could be found. For example, the following points are opened to further investigations : a) the establishment of optimal methods of dilution and succussion of test compounds, b) the testing of other compounds and also of commercial drug preparations, c) the exploration of effects of higher dilutions, beyond those utilized in this work, d) the study of other biological and biochemical events that could be affected by ultra-low doses of agonists or antagonists, such as changes of ionic fluxes, of membrane permeability, of gene expression, etc.

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