Simultaneous assay for oxidative metabolism and adhesion of human neutrophils: evidence for correlations and dissociations of the two responses

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Abstract: An assay method for the simultaneous evaluation of the oxidative metabolism and adherence of human neutrophils is described, together with certain specific applications. Incubations were performed in serum-coated microtiter plates, where oxidative metabolism was measured as O_2^- release and, after washing out the nonadherent cells, the adhesion was measured as activity of acid phosphatase. Three agonists tested in this system—opsonized zymosan, concanavalin A, and *N*-formyl-methionyl-leucyl-phenylalanine—induced both activation of O_2^- release and cell adhesion, but the two functions had time course and dose dependence patterns that varied depending on the stimulant. Particularly with concanavalin A, O_2^- release and adhesion response were markedly dissociated; this lectin at low doses increased neutrophil adherence without triggering any O_2^- production, whereas at high doses it increased both O_2^- production and adherence. Anti-integrin monoclonal antibodies did not affect adhesion induced by low-dose concanavalin A but inhibited the adhesion induced by the other tested agonists. Adhesion and O_2^- production were also found to be differentially affected by the NADPH oxidase inhibitor diphenylene iodonium, the sulfhydryl reagent *N*-ethylmaleimide and the A_2 agonist adenosine, indicating that these neutrophil responses have various transductional pathways that also depend on the type of stimulus. *J. Leukoc. Biol.* 51: 329-335; 1992.

Key Words: *adhesion · superoxide anion · neutrophil activation · neutrophil inhibitors · phagocytosis*

INTRODUCTION

The ability of neutrophils to adhere to microvascular endothelium and extracellular matrix components and to produce oxygen-derived free radicals is regarded as essential in the inflammatory response and in the phagocyte-dependent host defense systems. The crucial role of these two neutrophil functions in protection against bacterial infections is well exemplified by the life-threatening infections that affect patients with neutrophils genetically deficient in the ability to produce oxygen-derived free radicals [13, 17, 24] or to adhere [2, 3, 25, 37].

It is well known that cell responses to various agonists and antagonists are influenced by the experimental conditions employed, that is, cell concentration, composition of the assay medium, and use of suspended cells or cells settled on surfaces. In particular, several investigators have noted that adherence to biological surfaces may profoundly affect the ability of neutrophils to respond with a respiratory burst to a variety of stimulatory agents [14, 16, 19, 22, 30]. Furthermore, these and other studies support the view that activation of the respiratory burst and increase in adherence are closely linked and interdependent phenomena.

The availability of methods for simultaneous measurement of the respiratory burst and the adherence of stimulated neutrophils under the influence of various agonists and antagonists may provide new insights into the mechanisms of activation and the relationships between these two responses and may be of great utility for diagnostic purposes.

In this report we describe a microplate assay method for the measurement, in the same assay system, of O_2^- release and adherence of neutrophils to serum-coated tissue culture plates. The method has been used to assess the effect of several agonists and antagonists of neutrophil activation.

The results show that activation of the respiratory burst and adhesion proceed as associated phenomena in some experimental conditions. However, from the results obtained with certain stimulatory agents and from studies using inhibitors of either of these functions, instances were found in which the two responses could be dissociated, suggesting that the two functions may be regulated by distinct activation and/or effector mechanisms.

MATERIALS AND METHODS

Materials

The chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), zymosan, Triton X-100, and *N*-ethylmaleimide (NEM) were purchased from Sigma Chemical Company, St. Louis, MO; cytochrome *c* and adenosine from Boehringer, Mannheim, Germany; concanavalin A (Con A) from Vector Laboratories, Burlingame, CA; and purified human albumin from Behring Institut, Marburg, Germany. Human copper-zinc superoxide dismutase (SOD) was a gift from Prof. J.V. Bannister (Cranfield Institute of Technology, Bedford, UK). Diphenylene iodonium (DPI) was kindly provided by Dr. A.R. Cross (Biochemistry Department, University of Bristol, UK). Fetal bovine serum (FBS) was

Abbreviations: Con A, concanavalin A; DPI, diphenylene iodonium; FBS, fetal bovine serum; fMLP, formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; H-GCMA, HBSS plus glucose, $CaCl_2$, $MgSO_4$, and albumin; αMM, α-methyl mannopyranoside; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; SOD, superoxide dismutase; STZ, serum-treated zymosan.

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from Flow Laboratories. Serum 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. The monoclonal antibody 60.3 was kindly provided by Dr. A. Dobrina (University of Trieste, Italy). Sterile 96-well microtiter plates with flat-bottomed wells (Linbro type) were from Flow Laboratories. Hanks' balanced salt solution (without calcium and magnesium) (HBSS) was from Gibco, 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 plasticware were used in all experiments, which were carried out, whenever possible, under a laminar-flow hood. Reagents were prepared using pyrogen-free water or 0.9% NaCl solutions.

Stock solutions of compounds were prepared and stored as follows: fMLP (10^{-4} M) was dissolved in dimethyl sulfoxide and stored at −20°C. DPI (1 mM) was dissolved in ethanol and stored at −20°C. Zymosan was prepared as described [28], opsonized with a pool of normal human sera, and stored in aliquots at −20°C at the concentration of 20 mg/ml. Working dilutions of fMLP, DPI, and serum-treated zymosan (STZ) were freshly prepared from stock solutions before each experiment. Con A (10 mg/ml), NEM (3×10^{-2} M), and adenosine (10^{-2} M) were dissolved in 0.9% NaCl and used on the day of the experiment. Dilutions of test compounds were made in 0.9% NaCl to a concentration exactly six times higher than that required in the assay. This was done because each agent is diluted six times in the final incubation mixture (see below).

Human neutrophils were prepared from ethylene EDTA-anticoagulated blood by centrifugation over Percoll gradients [28, 31]. Cells were finally suspended in HBSS supplemented with 5 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgSO_4$, and 0.2% human serum albumin (H-GCMA) at the concentration of 2.7×10^6/ml and kept at room temperature until use. Starting from 40 ml of blood, the yield ranged from 6×10^7 to 11×10^7 cells, and more than 98% of purified cells were neutrophils.

Microplate Coating

Preliminary experiments showed that neutrophils incubated in microplates spontaneously adhered to the bottom of the wells in less than 30 min and produced considerable amounts of O_2^-, thus preventing the possibility of studying the cell adhesion and metabolism upon addition of specific stimuli. Nonspecific activation was totally abolished by coating the microplate wells with serum. In order to use safe, sterile, and standardized serum preparations, FBS was used for this purpose. One hundred microliters or 50% FBS in phosphate-buffered saline (PBS) was 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 PBS using an automatic plate washer (Easy Washer 2, SLT Laboratories Instruments).

Assay of Superoxide Anion Production and of Adhesion

Superoxide anion was measured by the SOD-inhibitable reduction of ferricytochrome *c* [5] and adhesion by an enzymatic assay after removal of nonadherent cells by washing with an automatic plate washer (see below). 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) and the number of compounds to be tested. Preliminary experiments carried out with paired samples containing cytochrome *c* and cytochrome *c* plus SOD showed that the reduction of cytochrome *c* was totally inhibited by SOD. Therefore, the assays were carried out in the presence of cytochrome *c* only, and the production of O_2^- was calculated on the basis of the amount of reduced cytochrome *c*. Operatively, immediately after coating with FBS, the wells were supplemented with 25 μl of the test compounds diluted in 0.9% NaCl at concentrations six times higher than the final concentration or, in controls, with 25 μl of 0.9% NaCl or with 25 μl of dilutions, in 0.9% NaCl, of the solvents used to prepare the test compounds. The plate was then brought to 37°C, and 75 μl of the neutrophil suspension (2×10^5 cells), prewarmed at 37°C, was added to each well using a multichannel pipette. The plate was incubated for 10 min at 37°C, and the wells were then rapidly supplemented with 50 μl of a solution (prewarmed at 37°C) of H-GCMA containing 0.45 mM ferricytochrome *c* (final concentration 0.15 mM) plus the stimulatory agent at a concentration three times higher than the final concentration. In all procedures, care was taken to avoid cooling the plate when it was taken from the incubator for additions and readings. At the end of the incubation, the plates were rapidly transferred into a microplate reader (Reader 400, SLT Laboratories Instruments) and the reduction of cytochrome *c* was measured at 550 nm using 540 nm as a reference wavelength to avoid interference due to light scattering. Quantitation of absorbance of standard amounts of reduced and oxidized cytochrome *c* established that the absorbance of 1 nmol of reduced cytochrome *c*, at 550 nm minus 540 nm, was 0.037 optical density units.

For adherence measurements, immediately after reading cytochrome *c* reduction, the plate was transferred to the automatic washer and subjected to two washing cycles with PBS at room temperature. Washing was carefully calibrated in order to obtain optimal sensitivity and reproducibility of adhesion measurements. Each cycle was carried out as follows: aspiration, filling by gentle jet for 1.5 s, and, after about 30 s, aspiration. Adherent cells were quantitated by measuring the membrane enzyme acid phosphatase. Seventy five microliters of 0.15 M acetate buffer, pH 5.3, containing 0.2% Triton X-100 was dispensed into the wells. After 5 min at room temperature, 75 μl of the 0.15 M acetate buffer, pH 5.3, containing the substrate (10 mM *p*-nitrophenyl phosphate) was added. After incubation at room temperature for 20 min, the reaction was stopped by the addition of 100 μl of 2 N NaOH. The *p*-nitrophenol produced in the reaction was measured with the microplate reader at 405 nm. The percentage of adherent cells was calculated on the basis of a standard curve obtained with known numbers of neutrophils.

RESULTS

Kinetics and Dose Dependence of Respiratory Burst and Adhesion

A set of experiments was preliminarily carried out to establish the optimal conditions for the measurement of O_2^- release and adhesion in our assay system. Both the SOD-inhibitable reduction of cytochrome *c* (assay of O_2^- release) and the acid phosphatase activity (assay of adhesion) were linear in a range of cell concentrations varying from 2×10^4 to 3×10^5 neutrophils/well. All subsequent experiments were therefore performed using 2×10^5 cells/well.

Figure 1 shows the time course of O_2^- release and adhesion of neutrophils exposed to fMLP, STZ, and Con A. All three agonists stimulated both O_2^- release and adhesion, but with differences in the kinetics of the two responses. The fMLP (Fig. 1A) induced a rapid increase in O_2^- release that plateaued after 10 min of incubation. Adhesion, in the corresponding experiments, increased more slowly, reaching a maximum of about 30% adherent cells after 30 min of incubation. The release of O_2^- induced by STZ (Fig. 1B) started after a 10-min lag, increased up to 40–60 min, and then tended to level off. The adhesion showed a kinetic trend similar to that of O_2^- release during the first 30 min of incubation. From 30 min onward a slight decrease in adhesion was observed, probably due to cell detachment. With Con A, O_2^- release and adhesion showed a time course profile different

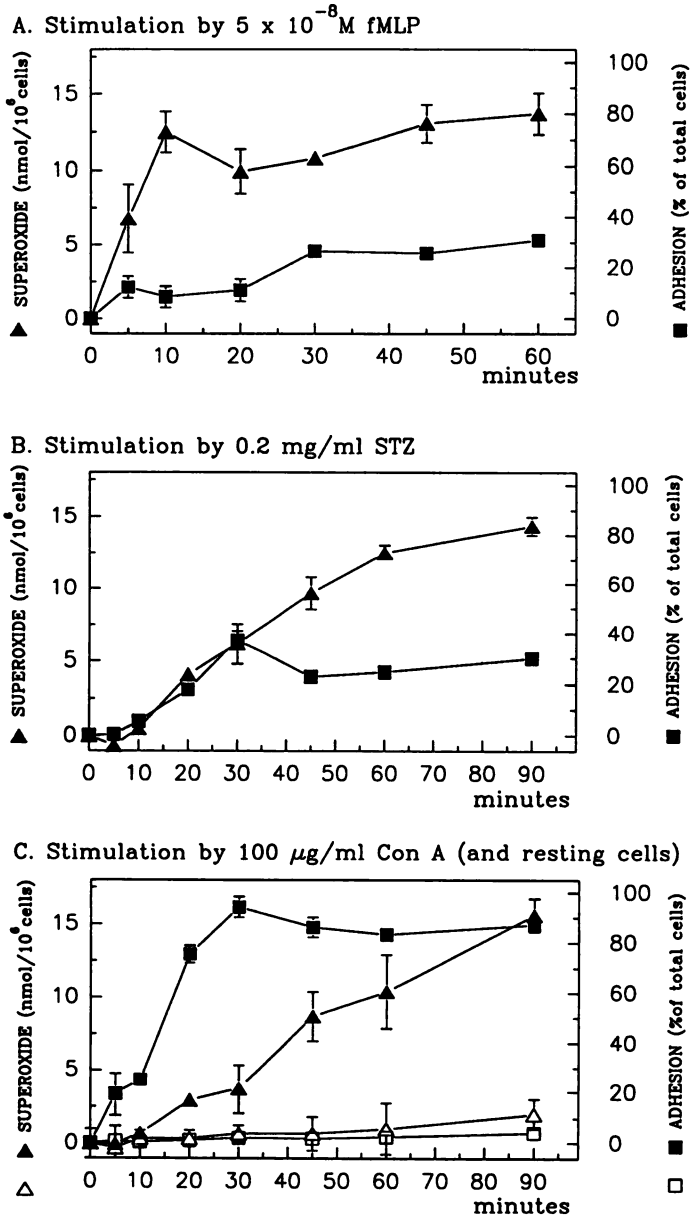

Fig. 1. Time course of O_2^- production (▲) and adhesion (■) of human neutrophils stimulated with fMLP (A), STZ (B), and Con A (C). Open symbols (C): cells incubated in the absence of stimulants. The reactions were carried out in different plates according to the times of incubation. Washing of the plates for quantitation of adherent cells was accomplished in 10 to 30 after measurment of O_2^- production. Data are means ± SD of triplicate wells from one of three experiments.

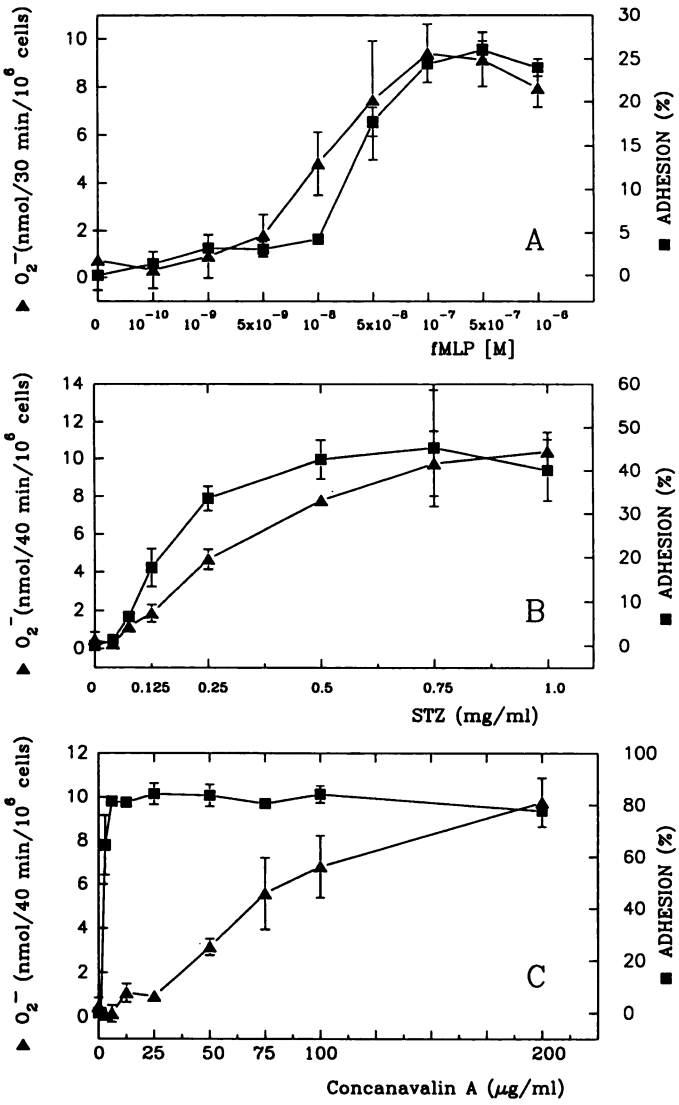

Fig. 2. Dose-response curves of the effect of fMLP (A), STZ (B), and Con A (C) on O_2^- production (▲) and adhesion (■) of human neutrophils. Data are means ± SD of triplicate wells from one of five experiments.

from that observed with the other stimuli. In fact, as shown in Figure 1C, Con A-induced O_2^- release was appreciable after 20 min of incubation and increased linearly up to 90 min, while adhesion was much more rapid in onset, maximal after 30 min of incubation, and so massive as to involve about 100% of cells. Figure 1C also shows that in the absence of stimuli, spontaneous O_2^- release and adhesion are very low or absent. This might be important for the detection of small increases in O_2^- release and/or adhesion, such as those possibly induced by stimuli at low doses or by weak stimulatory agents.

The relationship between superoxide production and adhesion was further explored in dose-response experiments (Fig. 2). On stimulation of the cells with fMLP and STZ, O_2^- release and adhesion exhibited similar, but not perfectly overlapping, dose-response curves. With fMLP (Fig. 2A) the O_2^- release curve was slightly shifted to the left with respect to the adherence curve, whereas with STZ (Fig. 2B) the O_2^- release curve was slightly shifted to the right. With Con A, the dissociation of the two cell functions was much more pronounced (Fig. 2C): a marked adhesion response was observed at doses of the agonist (3.15–25 μg) that did not trigger

any O_2^- release. Adhesion was already maximal at 6.25 μg/ml Con A, but O_2^- release was only detectable at 50 μg/ml Con A and increased as a function of Con A concentration, reaching a maximum at 200 μg/ml. In four separate experiments, the median effective doses (ED_{50}) for Con A-induced O_2^- release and adhesion were 55 ± 15 μg/ml and 4.2 ± 2.6 μg/ml, respectively.

Effects of Antagonists and Inhibitors

These results indicate that agonist-induced increase in adherence and activation of the respiratory burst may occur independently. To better understand the relationship between adhesion and activation of the respiratory burst, we studied the effect of a series of agents known to interfere with these responses at different levels of the activation cascade. Table 1 shows the effects of monoclonal antibody 60.3, known to inhibit the CD11/CD18 (β2 integrin)-dependent adhesion of neutrophils to various substrates [21], and of α-methyl mannopyranoside (αMM), an inhibitor of Con A binding to neutrophil plasma membranes [33]. Monoclonal antibody 60.3 and αMM differentially affected O_2^- release and adhesion, depending on the stimulatory agent used. The monoclonal antibody strongly inhibited the adhesion induced by fMLP and STZ but had only a slight inhibitory effect on the adhesion induced by low doses of Con A (5 μg/ml), while producing roughly 60% inhibition of the adhesion induced by 100 μg/ml of Con A. Under the same conditions the monoclonal antibody had no effect on the fMLP- and Con A-induced O_2^- release and only partially affected (50% inhibition) the O_2^- release induced by STZ. The partial inhibitory effect on STZ-induced O_2^- release by the anti-CD11/CD18 monoclonal antibody is in agreement with the fact that recognition of opsonized zymosan by neutrophils partly occurs via CD11/CD18 integrin receptors and partly via glucan and mannan receptors [8, 40]. With αMM, the responses to fMLP and STZ were unaffected but both the adhesion and O_2^- release induced by Con A were almost completely inhibited; the residual adhesion observed with 100 μg/ml Con A is probably due to incomplete competition with such a high dose of the lectin.

The relationship between oxidative metabolism and adhesion was also investigated by incubating the cells with agents known to inhibit the activity and/or the activation of the O_2^--generating oxidase [11]. Figure 3 shows that DPI, a direct inhibitor of the flavin moiety of the NADPH oxidase [12], blocked both fMLP- and STZ-induced O_2^- release in a dose-dependent manner. In contrast, adhesion was either unaffected by the drug or, with STZ, even slightly increased in the concentration range 10^{-8} to 10^{-6} M. The sulfhydryl reagent NEM inhibited the fMLP-induced O_2^- release and adhesion in a dose-dependent manner (Fig. 4A). However, O_2^- release was more sensitive to inhibition by NEM than adhesion, as indicated by the different ID_{50} values for the two responses: 7.5×10^{-6} M NEM for adhesion and 2×10^{-6} M for O_2^- release. STZ-induced adhesion (Fig. 4B) was very sensitive to NEM inhibition, with a dose dependence curve similar to that observed for inhibition of fMLP-induced adhesion. In contrast, STZ-induced O_2-release was much more resistant to inhibition by NEM and was slightly inhibited only at the highest inhibitor concentrations used.

Figure 5 shows the effect of adenosine, a nucleotide that has been shown to inhibit O_2^- production by neutrophils [9, 39], with a mechanism probably involving an increase in the intracellular levels of cAMP [10, 15]. In our conditions, adenosine inhibited O_2^- release induced by fMLP but had no effect on STZ-induced O_2^- release. The nucleotide partially inhibited the adhesion response to both stimulants, with similar dose-response curves.

TABLE 1. Effect of Monoclonal Antibody 60.3 and α-MM on O_2^- Production and Adhesion of Neutrophils Stimulated by Various Agents[a]

Agent	Effect	Experiment	Control	60.3	α-MM
No stimulants	O_2^-	A	0.5 ± 0.3	0.7 ± 0.2	0.7 ± 0.4
		B	0.8 ± 0.3	0.7 ± 0.1	0.4 ± 0.4
	Adhesion	A	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.1
		B	4.0 ± 0.3	2.9 ± 0.5	4.0 ± 0.4
fMLP (5×10^{-8} M)	O_2^-	A	11.8 ± 0.1	10.9 ± 1.1	10.1 ± 0.8
		B	34.7 ± 1.7	28.9 ± 5.4	31.5 ± 4.3
	Adhesion	A	9.9 ± 1.2	0.1 ± 0.2	7.7 ± 1.3
		B	19.7 ± 4.1	2.7 ± 0.2	16.0 ± 3.7
STZ (0.25 mg/ml)	O_2^-	A	9.2 ± 1.9	4.2 ± 0.6	9.2 ± 0.6
		B	7.3 ± 1.4	5.5 ± 1.0	7.5 ± 1.0
	Adhesion	A	23.8 ± 5.5	0.4 ± 0.3	22.9 ± 1.8
		B	19.6 ± 3.2	3.7 ± 1.6	20.1 ± 1.1
Con A (5 μg/ml)	O_2^-	A	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.3
		B	1.2 ± 0.1	1.9 ± 0.3	0.7 ± 0.4
	Adhesion	A	58.0 ± 4.0	55.2 ± 4.4	0.2 ± 0.0
		B	71.2 ± 4.6	57.3 ± 6.4	4.5 ± 0.4
Con A (100 μg/ml)	O_2^-	A	6.5 ± 0.2	9.2 ± 1.9	0.9 ± 0.4
		B	7.6 ± 1.6	12.2 ± 1.5	0.6 ± 0.3
	Adhesion	A	95.1 ± 1.6	43.0 ± 6.1	11.0 ± 4.2
		B	98.8 ± 1.4	40.1 ± 2.3	10.4 ± 1.3

[a]Neutrophils were preincubated for 10 min in the absence and in the presence of 20 μg/ml of monoclonal antibody 60.3 or of 50 mM α-methyl mannopyranoside (αMM), and the indicated stimulants were then added according to the procedure described in the methods. The incubation was carried out for 30 min. Data for O_2^- (nmol/30 min/10^6 neutrophils) and adhesion (% of adherent neutrophils) are means ± SD of triplicate determinations from two independent experiments (A and B).

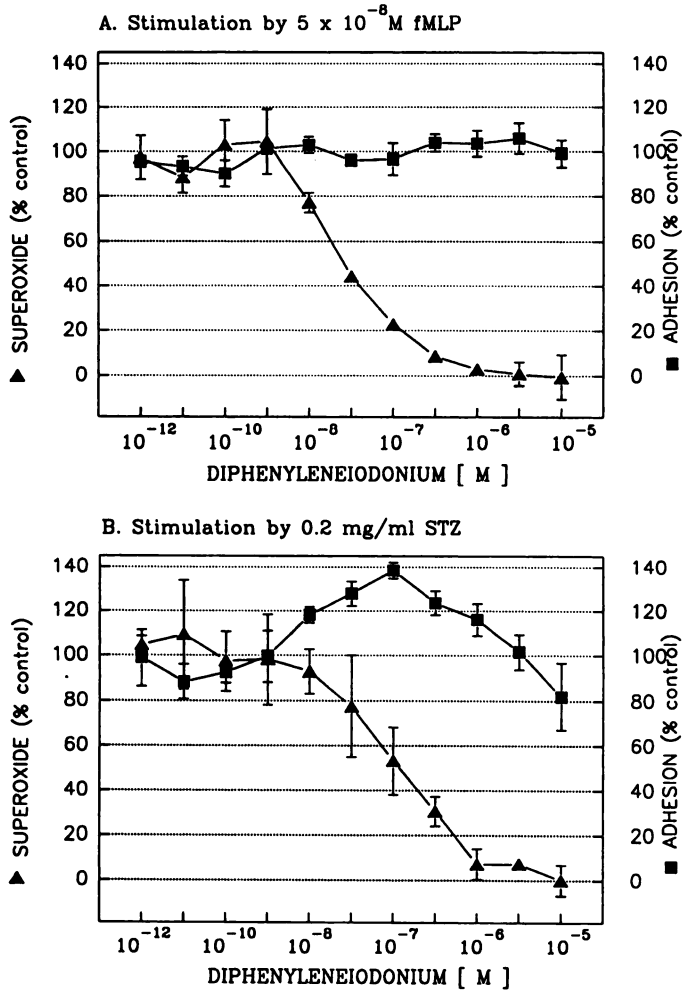

Fig. 3. Effects of the NADPH oxidase inhibitor diphenylene iodonium on O_2^- production (▲) and adhesion (■) of human neutrophils. Data are means ± SD of triplicate wells from one of three experiments.

DISCUSSION

Neutrophil-dependent immunological surveillance is essential for the destruction of invading microorganisms but also contributes to tissue injury in a variety of pathophysiologic conditions. Several neutrophil functions, including microvascular margination, chemotaxis, antibody-dependent cell cytotoxicity, phagocytosis, and bacterial killing, depend on the ability of these cells to adhere and to respond with a respiratory burst [1, 4–6, 23, 38].

In this paper we have described an assay method for the simultaneous assessment of adhesion and activation of the respiratory burst of neutrophils and have shown some of its applications. The assay is sensitive and versatile, allowing performance of a two- to three-plate experiment starting from a relatively small amount of blood (40 ml), which can easily be obtained as a supplemental sample from blood donors. The assay of O_2^- in microplates is similar to that reported by others [22, 28, 32], with the differences that here (1) double wavelength reading (550 and 540 nm) has been adopted in order to avoid any interference due to light scattering by the cells; (2) plates were routinely coated with FBS, a procedure that totally avoided artifactual cell activation by plastic surfaces; and (3) SOD was omitted from the standard assay mixtures for the reasons reported in the method section. Microplate assays for adhesion are mostly based on radioactive [29] or colorimetric [18] methods. Radioactive methods present obvious problems. A fundamental limitation of the colorimetric methods is that their use allows assessment only of qualitative variations in adherence and not of the absolute number of adherent cells [18]. Microassays for adhesion based on measurement of enzymes such as lactate dehydrogenase [7] or alkaline phosphatase [35] have been also proposed, but they are either more time-consuming [7] or less sensitive [35] than the acid phosphatase assay described here. Our method affords reliable and quantitative estimation of neutrophil adhesion with as few as 2×10^4 adherent neutrophils/well.

The biochemical mechanisms of activation and regulation of adherence and of the respiratory burst are very complex, involving a recognition apparatus, membrane events (lipid breakdown and synthesis, ionic and electric currents, activation of GTP-binding proteins, etc.), generation of intracellular messengers, and control of protein phosphorylation by kinases and phosphatases. Depending on the dose and on the type of agonists and antagonists, selected transduction pathways are turned on at variable intensity and duration. This ultimately leads to the modification of a particular biochemical system that operates on one or more cell responses. By these refined mechanisms various neutrophil functions including adhesion, aggregation, directional movement, phagocytosis, degranulation and production of oxygen-derived free radicals, are triggered in a coordinate and sequential fashion. Besides saving time and materials, the pos-

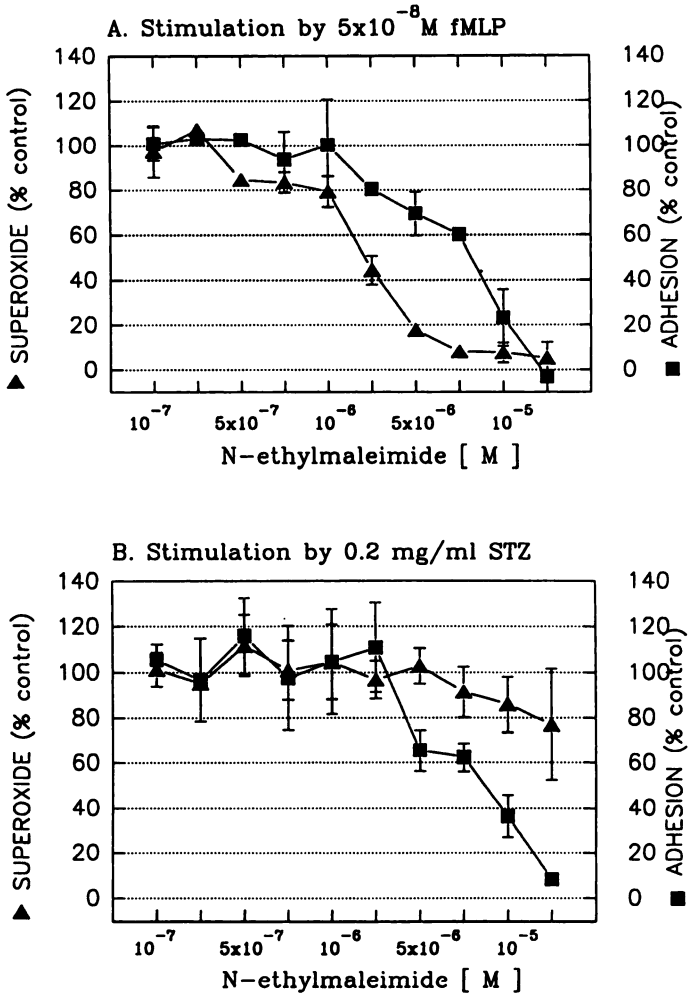

Fig. 4. Effects of the sulfhydryl reagent *N*-ethylmaleimide on O_2^- production (▲) and adhesion (■) of human neutrophils. Data are means ± SD of triplicate wells from one of three experiments.

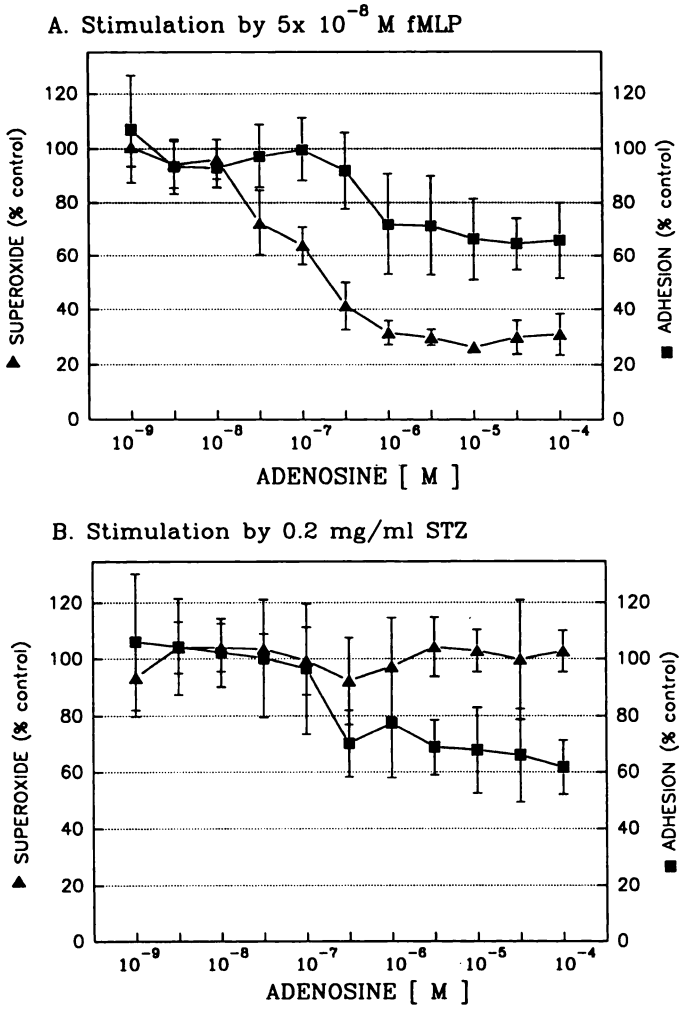

Fig. 5. Effects of adenosine on the O_2^- production (▲) and adhesion (■) of human neutrophils. Data are means ± SD of triplicate wells from one of three experiments.

sibility of simultaneously measuring two cell functions, namely adherence and O_2^- production, offers an opportunity to study the relationship between them. In this study we report several examples in which test compounds affect adhesion and metabolic activation in a differential manner. For example, Con A at low doses is a potent stimulus for adhesion and does not induce any O_2^- production, whereas at high doses it stimulates both responses (Fig. 2); fMLP and STZ stimulate both adhesion and O_2^- production, but the two responses are differentially sensitive to a monoclonal antibody directed against the common β chain of CD11/CD18 integrins. In fact, although adhesion was totally suppressed by the monoclonal antibody, O_2^- production induced by fMLP was unaffected and that induced by STZ was only partially inhibited by the monoclonal antibody. These results and the results of the experiments with Con A suggest that adhesion is neither necessary nor sufficient for activation of the respiratory burst and support the view that the signal transduction pathways for the different neutrophil responses may be experimentally separated and analyzed.

The classic view that adherence to substrates causes spreading of the cells in an attempt to phagocytose them ("frustrated phagocytosis") and therefore causes activation of the respiratory burst should be revised. This simultaneous activation may undoubtedly occur when neutrophils adhere to uncoated plastic wells or to other "activating" surfaces, but we have demonstrated here that the two phenomena are not directly correlated on a more physiological substrate (i.e. serum protein-coated surfaces) and with particular agonists (in this case Con A, but it is conceivable that other lectins may cause similar effects). It is possible that Con A, at least at low doses, binds to receptors that mediate adhesion but not the burst. Similar observations have been reported by Ginis and Tauber [19], who noted a sustained burst of superoxide when the neutrophils adhered to plastic but no activation when the adhesion was mediated by fibronectin. Because high doses of Con A also stimulate the respiratory burst, it follows that in these conditions an activating signal is transduced from membrane receptors to NADPH oxidase. This activation could result from cross-linking of Con A receptors or via other receptors of a glycoprotein nature. The fact that monoclonal antibody 60.3 slightly increased the metabolic activation induced by high doses of Con A (Table 1) indicates that in these conditions the antibody may act in synergism with Con A in stimulating the respiratory burst, suggesting that integrin-type adhesion molecules may be involved in the transduction of the activating signal by high doses of Con A. This interpretation is in agreement with the observation that various neutrophil receptors, including CD11/CD18 integrins, have affinity for Con A [34, 36].

The data obtained with inhibitors (Figs. 3-5) indicate that the test may be of value in studies of the possible effects of drugs on neutrophil functions, particularly in the fields of pharmacology of inflammation and immunomodulation. Demonstration of effects on one function, activation of the respiratory burst, in the absence of effects on a different function, adherence, as with DPI (Fig. 3), might be important to establish the specificity of the drug and also its action mechanism. In view of the dual role (defensive and destructive) played by neutrophils in many physiopathologic processes, it would be desirable to design drugs with increased specificity for selected cell functions. The differential sensitivity of the respiratory burst to inhibition by NEM depending on the agonist used (Fig. 4) is particularly puzzling. Our data suggest that (1) the effect of NEM on O_2^- production does not result from a direct inhibitory effect on the terminal oxidase of the respiratory burst, because if this were the case the O_2^- production induced by both fMLP and STZ should have been equally inhibited, and (2) the inhibitory effects on NEM do not depend on inhibition of receptor systems for fMLP or STZ, because if this were the case NEM should have inhibited both O_2^- release and adhesion to the same extent. It is known that multiple transduction mechanisms may activate the NADPH oxidase of neutrophils [26, 27] and therefore it is conceivable that in the sequence of events involved in the activation of the oxidase by fMLP (but not by STZ) some steps are very sensitive to blocking of essential sulfhydryl groups.

The finding that adenosine differentially affects adhesion and O_2^- production depending on the agonist used (Fig. 5) is of particular interest in view of the proposed physiological role of this compound [9, 20]. It is possible to speculate that the adenosine-induced inhibition of the respiratory burst triggered by chemotactic peptides may have a protective role against tissue damage caused by oxygen-derived free radicals produced by neutrophils exposed to chemotactic agents.

The method described in this paper may also have a number of applications in studies related to the diagnosis of clinically relevant neutrophil defects of either adhesion or respiratory metabolism, in the identification and functional characterization of surface molecules that mediate adhesion and signal transduction, and in the investigation of the mechanism involved in the activity and specificity of anti-inflammatory drugs.

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