

# Role of Cell Surface Contact in the Kinetics of Superoxide Production by Granulocytes

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**ABSTRACT** The complement-derived anaphylatoxin C5a and a putative analogue of bacterial chemotactic factor (*N*-formyl-methionyl-leucyl-phenylalanyl [fMLP]), as well as bacterial lipid A, all stimulate human granulocyte (PMN) adhesiveness and superoxide ( $O_2^-$ ) production in a concentration-dependent manner. Since attachment of particulate matter to the PMN membrane is an early event in the triggering of respiratory burst of these cells, we further examined how adherence might modulate the release of  $O_2^-$  induced by soluble mediators of inflammation. We found that both the quantity and kinetics of  $O_2^-$  production depend on prior attachment of the cells to a surface. In stirred suspensions of PMN, fMLP induces only a short burst (2.5 min) of  $O_2^-$  release associated with reversible PMN aggregation. The magnitude, but not the time course, of both these responses depend on the fMLP concentration. Unlike the short respiratory response of cells in suspension, PMN allowed to settle onto stationary petri dishes, then overlaid with fMLP, rapidly spread and attach to the surface where they remain and release  $O_2^-$  throughout the 60-min test period. Prolonged  $O_2^-$  release also follows fMLP stimulation in suspensions of PMN pretreated with cytochalasin B, in which case aggregation becomes irreversible during the 20-min burst. If fMLP is slowly infused into a suspension of cells at 37°C or if PMN are challenged at 0°C, and then warmed to 37°C,  $O_2^-$  release greatly decreases or becomes undetectable. Suspended PMN do not respond to a second challenge by the same stimulus regardless of the rate or temperature at which the first stimulus was added, a phenomenon formerly described as desensitization. However, if PMN challenged with fMLP in suspension undergo the short respiratory response and then are later placed in petri dishes, they adhere and resume pro-

duction of  $O_2^-$  without further stimulation. Chemotactic factor-induced adherence and  $O_2^-$  release of PMN on a surface is entirely independent of either the mode of activation or prior  $O_2^-$  release during preincubation in suspension. Human C5a also promotes PMN adherence and prolonged  $O_2^-$  release in petri dishes. Furthermore, lipid A increases  $O_2^-$  release and adherence of settled PMN, but fails to elicit either response from suspended PMN. These results indicate that cell surface contact plays an essential role in triggering the respiratory burst of PMN activated by soluble stimuli. This long-lasting  $O_2^-$  release by chemotactic factor-stimulated PMN may play a significant role in inflammatory reactions when PMN become adherent *in vivo*.

## INTRODUCTION

Oxygen radicals released during respiratory bursts of granulocytes (PMN) are believed to cause microbial killing and tissue damage at inflammatory sites. The biochemistry of these toxic radicals is well defined (1-5); however, the mechanisms for triggering (6-10) and terminating (11) the respiratory burst by PMN are largely speculative. Originally, it was recognized that the respiratory burst is associated with phagocytosis, but later studies demonstrated that simple contact of PMN with particles (12) or adherence to large opsonized surfaces induced the release of oxygen metabolites (13).

Because chemically unrelated compounds like concanavalin A, phorbol myristate acetate, fluoride, and digitonin induce PMN to release superoxide ( $O_2^-$ ),<sup>1</sup> "membrane perturbation" was tentatively considered

<sup>1</sup> *Abbreviations used in this paper:* C5a, the chemotactic peptide cleaved from the fifth component of complement during activation; fMLP, *N*-formyl-methionyl-leucyl-phenylalanyl; HMP, hexose monophosphate pathway;  $O_2^-$  superoxide ion; SOD, superoxide dismutase.

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sufficient to stimulate this response without the involvement of particles or surfaces. Soluble ligands have now been used extensively for PMN activation with the assumption that this method is preferable to using particulate stimuli and thereby avoiding the more complex and less definable cell-particle interaction (7–10, 14). That PMN stimulation by soluble factors is physiologically significant became clear after chemotactic factors such as *N*-formyl peptides (15–17) and endotoxins (18) were tested in this response and also induced the PMN respiratory burst.

Accordingly, we have used the chemotaxin *N*-formyl-methionyl-leucyl-phenylalanyl (fMLP) (15, 19) and bacterial lipid A (18) both to induce spreading and hyperadhesiveness of PMN. By varying conditions of the reaction with both of these reagents, we can demonstrate that O<sub>2</sub><sup>-</sup> production by PMN is dependent on cell-surface interactions. This mechanism may act as a common triggering event for initiating the respiratory burst induced by either particulate or soluble stimuli.

## METHODS

**Cells.** PMN were prepared from the blood of normal adult volunteers by means of Ficoll-Hypaque (Farmacia Inc., Uppsala, Sweden) density gradient separation. The resulting preparation had <0.5% contaminating mononuclear cells (18). After hypotonic lysis of erythrocytes and two washes in Gey's solution, the cells were suspended in an isotonic (300 mosmol; pH 7.4) buffer containing: 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.6 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 2 mM glucose, 5 mg/ml human serum albumin (electrophoretically pure, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA). For all O<sub>2</sub><sup>-</sup> release experiments, 100 μM cytochrome *c* (from horse heart, type IV, Sigma Chemical Co., St. Louis, MO) was added. In experiments involving measurement of the hexose monophosphate pathway (HMP), the PMN were suspended in autologous, heat-inactivated (56°C, 30 min) plasma as described (15, 19).

**PMN adherence.** As outlined in (15), 1-ml samples of 2–4 × 10<sup>6</sup> PMN were incubated in 10 × 35-mm petri dishes (Falcon Plastics, Oxnard, CA) at 37°C in 100% humidity and 5% CO<sub>2</sub>. Each stimulus was added in a volume of 1% that of the reaction mixture. At the time intervals indicated, each dish was washed thoroughly in isotonic NaCl, and the percentage of PMN attached to the surface of the dish was determined by measuring the myeloperoxidase content (15).

Some of the kinetic analyses of PMN adherence and O<sub>2</sub><sup>-</sup> production were performed in a 37°C thermostated room. The unstimulated PMN were allowed to settle onto the petri dishes for 10 min. Then, to obtain rapid saturation of fMLP receptors and even distribution of the reagent without mixing or resuspending the cells, 5 drops (2 μl each) of a highly concentrated solution of fMLP (10 mM) were carefully layered onto the air/liquid interface over a period of 5–10 s.

**O<sub>2</sub><sup>-</sup> production by PMN.** Release of O<sub>2</sub><sup>-</sup> was measured by superoxide dismutase (SOD; bovine, type I, Sigma Chemical Co.) inhibitable reduction of cytochrome *c*, using an extinction coefficient oxidized vs. reduced of 21.1 mM<sup>-1</sup> at 550 nm. Fresh SOD (10 μg/ml) inhibited cytochrome *c* reduction

by stimulated cells, whereas boiled SOD was ineffective. The enzyme had no inhibitory action on PMN adherence.

The kinetics of O<sub>2</sub><sup>-</sup> release from PMN in suspension were calculated from the change in absorbance at 550 nm as continuously monitored during stirring (900 rpm) in a thermostatically controlled (37°C) cell compartment. To avoid artefacts caused by changes in light scattering, a dual-wavelength spectrophotometer (Shimadzu Seisakusho Ltd., model UV 300, Kyoto, Japan) was used, and the reference wavelength was set at the isobestic point of 540 nm. The cells were preincubated for 10 min at 37°C before addition of the stimuli. However, in some experiments the stimuli were added at 0°C, and the PMN were subsequently placed in the photometer. Absorbance was measured continuously as the temperature rose to 37°C.

In the absence of a stimulating agent, cytochrome *c* reduction was virtually absent (<0.2 nmol cytochrome *c* reduction/min for 5 × 10<sup>6</sup> PMN). It is important to note that, at maximal levels of chemotactic factor, O<sub>2</sub><sup>-</sup> release varied (up to 250%) among different batches of cells, as shown by others (17). Since we were particularly interested in the kinetics of the response, which were always superimposable, these variations in amounts released presented no problem. Additionally, identical fresh cell preparations were used to generate each value for quantitative comparisons.

For measurements of O<sub>2</sub><sup>-</sup> production on petri dishes, PMN were incubated as described in the adherence assay and the supernates were withdrawn into precooled (0°C) tubes. Residual cells were sedimented by centrifugation, and the reduction of cytochrome *c* was measured by using the optical difference spectrum (from 560 to 540 nm) between supernates and the buffer. With this technique, even 1% of reduced cytochrome could be accurately measured.

**Glucose oxidation by the HMP.** The HMP activity of PMN was determined by measuring generation of <sup>14</sup>CO<sub>2</sub> from [1-<sup>14</sup>C]glucose as described (18, 19) under two different incubation conditions: (a) with cells kept in stationary petri dishes and (b) with cells held in suspension. The procedures differed only in that, for the latter, 1-ml portions of PMN were placed in plastic tubes and agitated.

PMN aggregation was measured as described (20) by using a platelet aggregometer (dual channel, model 314 Payton Associates, Inc., Buffalo, NY). The *N*-formylated chemotactic peptide fMLP was obtained from Bachem, AG., Bubendorf, Switzerland. Human C5a, the complement-derived chemotactic glycopolyptide of known primary structure (21), was isolated as previously described (22). The purity of this C5a was assessed by amino acid analysis and migration as a single narrow band on cellulose acetate after electrophoresis at pH 8.6.

Lipid A (the generous gift of Dr. Chris Galanos, Max Planck Institute, Freiburg, GFR) prepared from endotoxin of *Salmonella minnesota* R595 (23) was used as described (18). Cytochalasin B (Sigma Chemical Co.) dissolved in dimethyl sulfoxide (0.1% vol/vol final concentration) was added 5 min before adding the cellular stimuli. Statistical analysis of the results were done by the unpaired Student's *t* test and the method of least squares. All experiments were performed in triplicate and repeated at least three times. Results are expressed as mean ± SD.

## RESULTS

### *Stimulation of HMP activity by fMLP*

At 37°C, fMLP induced PMN to spread and attach to plastic surfaces, decreased locomotion, enhanced

enzyme release and stimulated a respiratory burst in a highly dose-dependent manner, as previously shown (15). However, when the stimulus was added at 0°C, and the cells were subsequently incubated at 37°C for 40 min in a shaking water bath, no significant increase of the HMP activity ensued (Fig. 1, curve 1). This finding could account for failure of other researchers to observe HMP enhancement by fMLP (24). When PMN were agitated and stimulated at 37°C, a significant, but unimpressive, threefold enhancement occurred with maximal fMLP concentrations (Fig. 1, curve 2).

Our former result from routinely adding fMLP to the PMN at 0°C (15) then incubating the mixture in petri dishes at 37°C, which yielded spreading and enzyme release, indicates that responses to chemotactic factor other than HMP activity are retained by the cell even when the stimulating agent is added at low temperature. However, adherence and enzyme release were measured when PMN were incubated in petri dishes (15), but HMP activity was assessed by incu-

bating PMN in shaking tubes. Thus, we now examined whether incubation conditions were responsible for our observations rather than a true separation between HMP activation and PMN adherence or enzyme release. When cells were incubated in petri dishes for 40 min at 37°C after a prior treatment with fMLP at 0°C, the basal HMP activity was unaffected by the difference in incubation conditions (Fig. 1, curve 3), but an impressive 20-fold increase occurred when 1  $\mu$ M fMLP was present. Not only did HMP activation occur under these conditions, but the magnitude of the response was much higher even when compared with suspended cells challenged with fMLP at 37°C. PMN in suspension and on plates responded to fMLP over the same concentration range. Moreover similar dose-response patterns characterized fMLP-induced aggregation, adherence, and O<sub>2</sub><sup>-</sup> release by the cells (data not shown), confirming published results (15-17, 25). The marked increase in HMP activity of the cells incubated in petri dishes could mean that fMLP-induced PMN adherence plays a causative role in the related

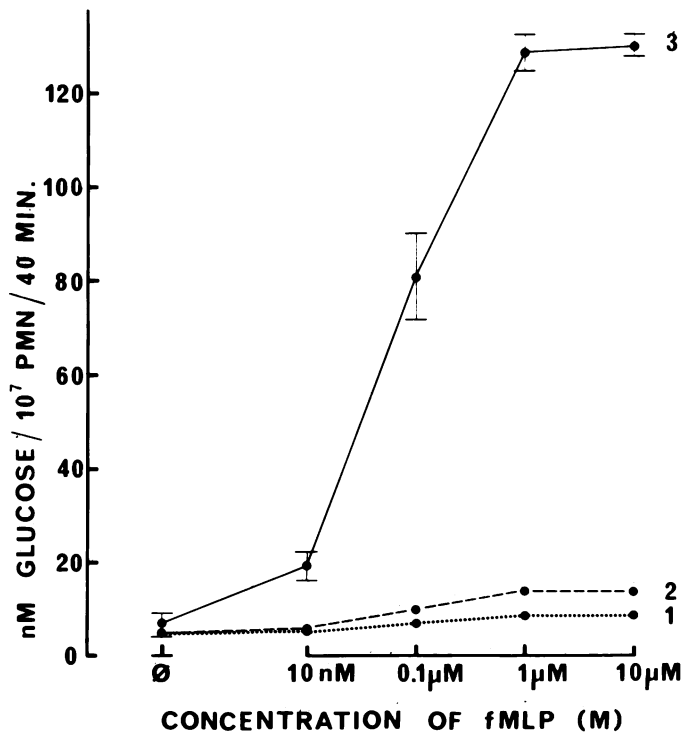


FIGURE 1 Dose-dependent stimulation of HMP in human PMN exposed to fMLP. The PMN ( $5 \times 10^6$ /ml) suspended in autologous plasma and after addition of the fMLP were incubated for 40 min at 37°C. (1) Dotted line: fMLP was added at 0°C and the reaction vessel was constantly agitated during incubation at 37°C. (2) Dashed line: fMLP was added after preincubation for 10 min at 37°C. The tubes were agitated throughout the incubation period. (3) Solid line: fMLP was added to PMN at 0°C and incubated in petri dishes at 37°C. Results are expressed as mean  $\pm$  SD of each data point performed in triplicate. Where not indicated, the SD is <10% of the mean. Results were similar when plasma-free buffer was used.

$O_2^-$  release; therefore, we next measured the kinetics of  $O_2^-$  production coincident with PMN aggregation and adherence.

### $O_2^-$ production by stirred cells in suspension

**Kinetics of  $O_2^-$  release and PMN aggregation.** The burst of  $O_2^-$  release was induced by fMLP after a lag period of 8–10 s (Fig. 2, curve 1), confirming prior reports (16, 26). The rate and magnitude of the response are dose dependent; however, the duration and lag period were independent of the fMLP concentration (Fig. 2, curve 2). After 1–1.5 min, the rate of  $O_2^-$  release declines and after 2.5 min no further release followed (Fig. 2, curves 1 and 2). Once fMLP was added, the PMN aggregated within  $\sim 5$  s (Fig. 3 A). Aggregation reached a maximum within 1–1.5 min, after which the cells began to deaggregate. As noted for  $O_2^-$  release, the time course of PMN aggregation was not dose dependent (data not shown).

**Influence of stimulus presentation on  $O_2^-$  production.** In agreement with the HMP data,  $O_2^-$  was not released after adding fMLP to the cells at  $0^\circ\text{C}$  (Fig. 2, curve 3); a similar result was reported by Lehmeier et al. (16). When fMLP was slowly infused at a rate of 20 pmol/ml per min for a period of 15 min, as described by Sklar et al. (26),  $O_2^-$  release decreased to  $\sim 7\%$  of that elicited by a bolus injection of 300 nM fMLP.

**PMN “deactivation”.** Cells stimulated by suboptimal (50 nM) or maximal (1  $\mu\text{M}$ ) concentrations of fMLP responded to a subsequent higher (10  $\mu\text{M}$ ) dose of the stimulant with a correspondingly reduced (Fig. 2, curve 2) or negligible (Fig. 2, curve 1)  $O_2^-$  production. This phenomenon has been called “deactivation” (17, 27). Of importance, even without prior  $O_2^-$  production, we observed no response to a second stimulation if fMLP was first added at  $0^\circ\text{C}$  (Fig. 2, curve 3) or infused slowly (data not shown). Like  $O_2^-$  production, aggregation was also negative after subsequent stimulation, confirming published results (28).

**Effect of cytochalasin B pretreatment.** Cytochalasin B enhanced both chemotactic factor-induced  $O_2^-$  release (12, 16, 17) and PMN aggregation (20, 25). As depicted in Fig. 3 the initial maximal rate of  $O_2^-$  production and PMN aggregation both increase three- to fourfold. Interestingly,  $O_2^-$  production remained elevated and aggregation was essentially irreversible over a period of 20 min, in marked contrast to the reversible stimulation observed in the absence of cytochalasin B. Our results differ from those of other workers, who found either an increased but limited burst (16), or a continuous response without an increase in the initial rate (17). Underestimation of  $O_2^-$  production due to a simultaneous decrease in light scattering of aggregated cells might account for this discrepancy, particularly if a detection system other than the dual wavelength method is used.

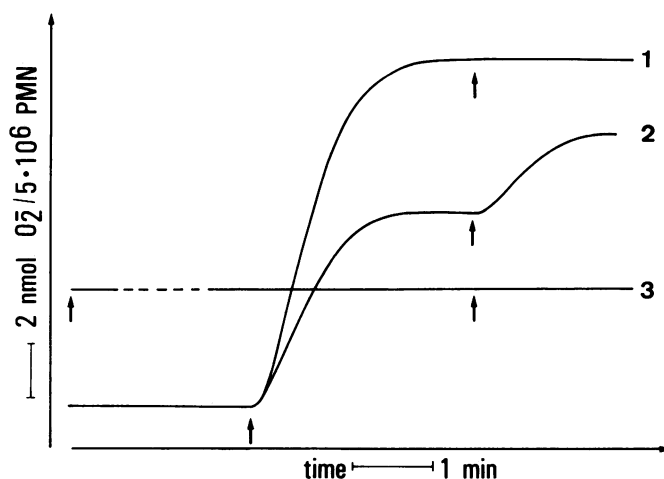


FIGURE 2 Kinetics of  $O_2^-$  release from stirred cell suspensions. (1) First arrow indicates when 1  $\mu\text{M}$  fMLP was added, second arrow indicates addition of 10  $\mu\text{M}$  fMLP. (2) First arrow indicates when 50 nM of fMLP was added, second arrow indicates addition of 10  $\mu\text{M}$  fMLP. For (1) and (2) the fMLP was added to cells preincubated for 10 min at  $37^\circ\text{C}$ . (3) First arrow indicates when 1  $\mu\text{M}$  of fMLP was added at  $0^\circ\text{C}$ , second arrow indicates that 10  $\mu\text{M}$  fMLP was added after incubation for 10 min at  $37^\circ\text{C}$ . The interrupted line indicates that no increase in  $O_2^-$  production was recorded during the incubation period of 10 min. Curves were traced directly from the spectrophotometric recordings. Results were repeated 10 times using four different cell preparations that provided identical kinetics.

### *O<sub>2</sub><sup>-</sup> release by adherent cells*

**Kinetics.** Stimulation of PMN, previously settled onto petri dishes, resulted in rapid and almost irreversible attachment of the cells (Fig. 4 A). The initial rate of O<sub>2</sub><sup>-</sup> release by these cells remained unchanged compared with responses of suspended cells not pretreated with cytochalasin B. However, cells in suspension ceased to release O<sub>2</sub><sup>-</sup> after 2.5 min (Fig. 4 B, inset), whereas PMN on dishes continued to produce O<sub>2</sub><sup>-</sup> throughout the observation period of 60 min (Fig. 4 B). Similarly, continuous and linear release of O<sub>2</sub><sup>-</sup> followed when fMLP was added at 0°C to suspended PMN later incubated in dishes at 37°C, except that the initial rate was slower (data not shown).

**Influence of the mode of activation.** We next examined how the rate or temperature at which fMLP was added influenced PMN adherence and O<sub>2</sub><sup>-</sup> production in petri dishes (Table I): Stirred cells were preincubated at 37°C under conditions identical to those used for kinetic experiments. (1) No chemotactic factor was added to the buffer control; (2) 250 nM fMLP was added to the cells after 10 min, when the temperature of the cell suspension had reached 37°C; (3) 250 nM fMLP was added to cells at 0°C and the cells were subsequently warmed to 37°C while stirred; and (4) fMLP was slowly infused at a rate of 20 pmol/min at 37°C to obtain a final concentration of 250 nM fMLP. After a preincubation period of 25 min, the cell preparation was divided into petri dishes and incubated 40 min longer. It is important to note that at the time when the cells were added to the dishes, all of the cells had ceased releasing O<sub>2</sub><sup>-</sup>. As shown in the previous section the amount of O<sub>2</sub><sup>-</sup> released during the preincubation period differed according to the mode of activation, although the final concentration of fMLP were identical.

As seen in Table 1, the cells exposed to fMLP adhered to the dishes and released O<sub>2</sub><sup>-</sup> in large amounts. In addition, stimulated adherence and O<sub>2</sub><sup>-</sup> release were identical regardless of the mode of activation. Moreover, O<sub>2</sub><sup>-</sup> released during the activation process did not influence adherence or O<sub>2</sub><sup>-</sup> release on petri dishes.

### *Bacterial lipid A-induced O<sub>2</sub><sup>-</sup> production*

The bacterial product lipid A has multiple effects on PMN functions, including adherence (18). As de-

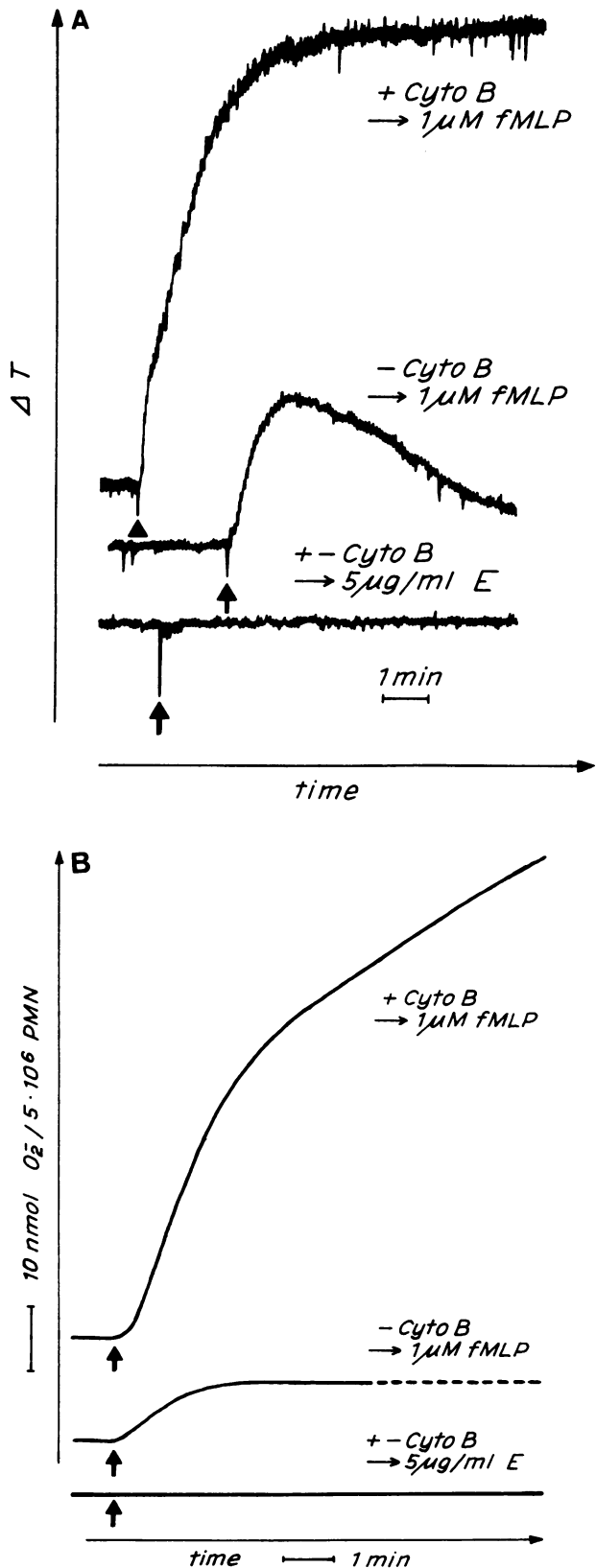


FIGURE 3 Response of stirred PMN to fMLP and endotoxin (E) at 37°C. (A) PMN aggregation. (B) Kinetics of O<sub>2</sub><sup>-</sup> release. The cells were preincubated for 5 min at 37°C with (+) and without (-) 5  $\mu\text{g/ml}$  cytochalasin B. Arrow indicates when fMLP or lipid A was added. Upper and middle curves represent aggregation induced by 1  $\mu\text{M}$  fMLP with and without cytochalasin B pretreatment, respectively. Lower curves show lack of PMN aggregation induced by 5  $\mu\text{g/ml}$  endotoxin-derived lipid A. Curves were traced directly from spectrophotometer and aggregometer recordings.

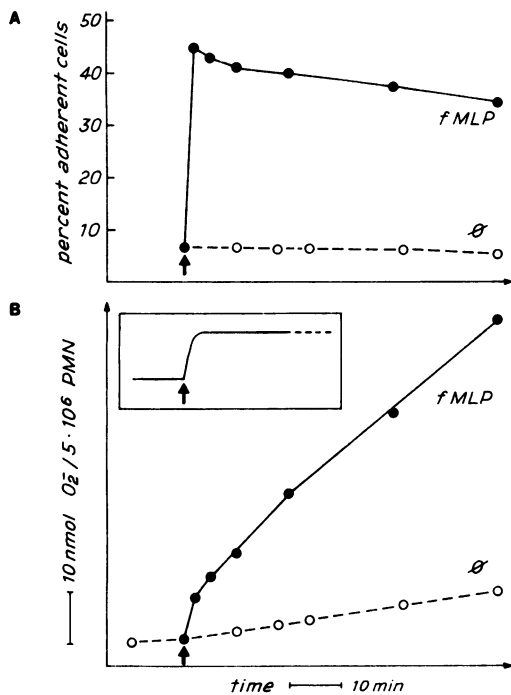


FIGURE 4 Influence of PMN adherence on  $O_2^-$  release after fMLP challenge. The PMN ( $4 \times 10^6/\text{ml}$ ) were preincubated in petri dishes for 10 min at  $37^\circ\text{C}$  before adding fMLP ( $100 \mu\text{M}$  final concentration). Arrows indicate stimulus addition, and the response is drawn as a solid line. Interrupted lines indicate the buffer control. Upper panel (A) contains the time course of PMN adherence. Lower panel (B) contains the time course of  $O_2^-$  release. Inset illustrates the corresponding response to the same concentration of fMLP by stirred cells from the same preparation. Points indicate the mean of triplicate determinations. SD was  $<10\%$  of the mean. For further experimental details see Methods.

pected in Fig. 3 and Table I,  $O_2^-$  production stimulated by lipid A also depended on PMN surface interaction. Lipid A added to stirred PMN induced neither aggre-

gation nor  $O_2^-$  release, even in the presence of cytochalasin B (Fig. 3 A and B). However, the respiratory burst observed after PMN settled and adhered to the surfaces of petri dishes was markedly augmented (Table I) by lipid A.

### PMN stimulation by C5a

Limited studies with the complement-derived chemotaxin C5a indicate a close association of PMN adherence and  $O_2^-$  release similar to that noted with fMLP. At a threshold level of  $10^{-9}$  M, C5a increased PMN adherence to petri dishes and  $O_2^-$  release (Fig. 5), both in a dose-dependent and highly correlated manner ( $r = 0.975$ ,  $P < 0.001$ ,  $n = 18$ ). The concentrations of C5a needed to induce  $O_2^-$  release and PMN adherence were above chemotactic levels, perhaps related to inhibition of PMN locomotion by high doses of this chemotactic factor as well as the inhibition of cellular migration induced by high doses of fMLP (15).

### DISCUSSION

This report clearly shows that the kinetics and quantity of oxygen radical production induced by either chemotactic factors or lipid A depend on prior attachment of the PMN to a surface. Cells kept in suspension and exposed to high concentrations of fMLP release relatively little  $O_2^-$  and lack responsiveness to a second fMLP challenge, regardless of the mode of activation or the amount of  $O_2^-$  initially produced. However, PMN stimulated and allowed to settle onto a surface release considerably more  $O_2^-$  and do so continuously over a prolonged period. Our observations indicate that the term "deactivation" of PMN might be misleading in a functional sense. It is clear that cells treated with mediator are, in fact, in an activated metabolic state, but the extent of release is defined by the incubation conditions. Thus, the general view that

TABLE I  
Influence of Mode of Activation on PMN Adherence and  $O_2^-$  Release

Preincubation condition	$O_2^-$ release				PMN adherence			
	Experiment 1		Experiment 2		Experiment 1		Experiment 2	
	nmol $\bar{x} \pm \text{SD} / 5 \cdot 10^6 \text{ PMN} / 40 \text{ min}$				%			
1. Buffer control	2.8	0.3	4.6	0.6	1.1	0.2	3.6	0.3
2. fMLP added at $37^\circ\text{C}$	46.4	1.3	55.6	1.5	44.7	2.1	58.9	3.1
3. fMLP added at $0^\circ\text{C}$	47.3	0.9	54.9	2.2	43.1	1.3	57.7	1.9
4. fMLP infused at $37^\circ\text{C}$	46.2	0.8	54.2	3.1	45.2	0.9	57.3	2.4
5. LA added at $37^\circ\text{C}$	58.7	2.1	44.4	3.1	63.5	3.3	46.3	3.1
6. LA added at $0^\circ\text{C}$	59.3	1.8	43.2	1.4	63.0	1.9	44.9	2.1

Stirred PMN were preincubated for 25 min at  $37^\circ\text{C}$ . fMLP (250 nM) and lipid A (LA,  $5 \mu\text{g}/\text{ml}$ ) were added as indicated in text. Thereafter cytochrome *c* ( $100 \mu\text{M}$ ) was added, and the cells were further incubated in petri dishes for 40 min. Data (mean  $\pm$  SD of triplicates) are derived from single experiments.  $P$  between 1 and 2–6  $<0.001$ ;  $P$  between 2 and 3 and 4, respectively  $>0.05$ ;  $P$  between 5 and 6, respectively  $>0.05$ .

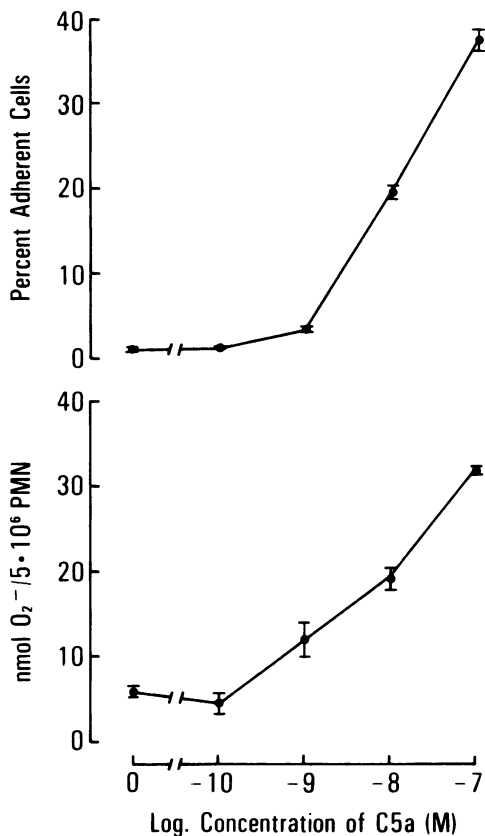


FIGURE 5 C5a-stimulated PMN adherence and  $O_2^-$  release are dose dependent when cells are incubated in petri dishes for 40 min at 37°C. C5a was added to the cells at 0°C. *Upper* panel portrays PMN adherence. *Lower* panel indicates  $O_2^-$  release. Each point represents the mean  $\pm$ SD for three determinations.

deactivation is a process limiting the inflammatory response (17, 26–28) should be reconsidered, particularly if release is dependent on cell-surface contact.

Of additional importance is the fact that adherence to and  $O_2^-$  production in petri dishes are dependent on the final dose of fMLP but independent of the rate at which the stimulus is added. Cells stimulated in suspension adhered and again release  $O_2^-$  when subsequently incubated in petri dishes, although they had previously ceased to produce  $O_2^-$  in suspension before settling onto the surface of the dish. The fact that adherence and  $O_2^-$  release in dishes was not affected by the rate of stimulation or the temperature at which the stimulus was added indicates that neither oxygen radicals nor other oxygen metabolites released during the activation process influence PMN adhesiveness or  $O_2^-$  release. We believe that  $O_2^-$  release resulting from PMN adherence is a physiologically relevant process, primarily because abrupt increases in the concentration of chemotactic factors are otherwise unlikely to

occur in vivo. Moreover, the respiratory burst in suspended PMN is small and short lived, even when the cells are activated rapidly; in contrast, a prolonged response is obtained from adherent PMN. Thus, during the inflammation reaction these cells may migrate along a gradient of chemotactic factors to the inflammatory site. The attracted cells may encounter an increased quantity of chemotaxin or possibly endotoxin and consequently become trapped at the lesion site as a result of increased adhesiveness (15, 18). When, thus, adhering to a surface, the PMN develop their full cytotoxic potential.

Data reported here might also relate to certain in vitro models of oxygen radical-dependent cytotoxicity. For example, PMN stimulated by chemotactic factor(s) were described as cytotoxic to other cells (29). We believe that continuous  $O_2^-$  release occurs in such models since it is difficult to imagine how the small, brief burst of oxygen radical release observed for suspended PMN can cause cytotoxicity.

The link between cellular spreading and oxygen radical production can be regarded as a biologically meaningful and economic mechanism, because the microenvironment created between the cell membrane and the target surface promotes accumulation of highly reactive oxygen compounds. In fact, this interface may embody properties very similar to those in the environment of phagolysosomes where free diffusion of oxygen radicals is also limited. Furthermore, one might speculate that responses elicited by both soluble and particulate stimuli depend on a common triggering event. Possibly adherence of PMN causes changes in membrane proteins that are involved in activating the oxygen radical system. This theory could also account for the considerable difficulty in defining the plasma membrane as the cellular site of  $O_2^-$  production (30–32), particularly since the  $O_2^-$ -generating system may function with greatest efficiency only when the  $O_2^-$ -generating complex is aggregated.

A close correlation is apparent between  $O_2^-$  production and aggregation of the PMN in suspension. Generation of  $O_2^-$  is associated with aggregation, and this release terminates when the cells deaggregate. Thus, PMN aggregation could be involved in initiating and maintaining  $O_2^-$  release. On the other hand, the mechanisms for terminating the inflammatory response of PMN may be controlled simply by their detachment. The molecular mechanism governing the effects of cytochalasin B pretreatment on the PMN response to chemotactic factors is unknown (33). Nevertheless, we showed that cytochalasin B increased both the initial rate of  $O_2^-$  release and the extent of aggregation. This continuous  $O_2^-$  release by cytochalasin B-treated cells might be related to the largely irreversible PMN aggregation much as  $O_2^-$  release is dependent on adherence, as reported here. However, such results fail to

demonstrate a causal relationship between these separate PMN functions. In fact, Lehmeyer et al. (16) showed that  $O_2^-$  release induced by fMLP occurs in the absence of  $Mg^{++}$ , whereas PMN aggregation depends on  $Mg^{++}$  (28, 34), indicating that the two reactions are separate. However, others have reported that  $O_2^-$  release as induced by fMLP is optimal only in the presence of both  $Mg^{++}$  and  $Ca^{++}$  (35).

In an elegant recent study of the PMN response to a phorbol ester (36), the authors examined the NADP<sup>+</sup>/NADPH ratio in individual cells by using a fluorescence-activated cell sorter. They concluded that increasing the concentration of phorbol ester increases the population of  $O_2^-$ -secreting cells but does not enhance  $O_2^-$  production of cells already activated. This is exactly the result one expects if cell aggregation is related to PMN stimulation, since only the aggregating subpopulation would release  $O_2^-$ .

Data currently available in the literature (28, 34) suggest that  $O_2^-$  release can be independent of PMN aggregation and/or adherence; however, the possibility that cell aggregation is involved in the stimulation of  $O_2^-$  release certainly warrants serious consideration, particularly since adherence of PMN to the petri dish surface or cell-particle interactions (12, 13) result in a respiratory burst. Additionally, many heterogeneous stimuli known to induce  $O_2^-$  production also lead to PMN aggregation and enhance cell stickiness (34, 37). Clearly, more work is required to determine whether cell adherence or aggregation is responsible for the stimulation of  $O_2^-$  release induced by soluble mediators.

Whatever the role of PMN aggregation, our results show conclusively that PMN adherence induced by soluble mediators can influence the kinetics of  $O_2^-$  production. Support for the hypothesis that  $O_2^-$  release depends on adherence comes from a recent study of Till et al. (38) whose electron micrographs illustrate that PMN remain in close contact with the pulmonary endothelium 15 min after complement is activated in vivo. In these experiments, damage to the microvasculature was inhibited by SOD and catalase. Consequently, contact of the PMN with the endothelium might indeed result in  $O_2^-$  release and constitute a major factor in subsequent cell damage in vivo. Further studies are required to answer the question of how the interaction between PMN and other tissue cells influence oxygen metabolism of activated granulocytes.

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