Chemotactic Factor Enhancement of Superoxide Release From Fluoride and Phorbol Myristate Acetate Stimulated Neutrophils

By Denis English, James S. Roloff, and John N. Lukens

Human neutrophils exposed to chemotactic concentrations of zymosan-activated serum (ZAS) and a formylated chemotactic peptide (FMLP, 10^{-6} to 10^{-4} M) were markedly enhanced in their ability to generate superoxide (O_2^-) upon stimulation with either sodium fluoride or phorbol myristate acetate (PMA). For both fluoride and PMA, enhancement was characterized by a decrease in the lag from stimulation to initiation of superoxide release and by an increase in the rate of superoxide generation—representing faster activation and increased activity of the O_2^- generating enzyme, respectively. Chemotactic concentrations of casein, normal serum, and casein-treated serum enhanced the activity, but not the rate of activation, of the fluoride-stimulated superoxide generating system. This effect on activity was not so impressive as that obtained with FMLP or ZAS. The mechanisms by which FMLP enhanced responsiveness to fluoride and PMA were found to be different. Optimal enhancement for fluoride-stimulated responses required extracellular Ca^{2+}. Extracellular glucose, but not extracellular Ca^{2+}, was required for enhancement of FMLP of PMA-stimulated responses. A similar glucose requirement could not be demonstrated for chemotactic peptide enhancement of the superoxide-generating system stimulated by fluoride. Fluoride and PMA apparently activate the neutrophil O_2^- generating enzyme by pathways that are not identical. However, responsiveness of the enzyme to both agents is susceptible to modulation by cellular responses to chemotactic peptides.

To reach sites of infection and inflammation, polymorphonuclear leukocytes pass through concentration gradients of chemoattractants. That the initial encounter of neutrophils with chemotactic factors may prime their responsiveness to stimuli they will reach at the distal end of the gradient is suggested by several observations. Allred and Hill¹ as well as Van Epps and Garcia² observed an enhancement by chemoattractants of phagocytically stimulated neutrophil chemiluminescence. McCall et al.³ reported that pretreatment of neutrophils with 10^{-6} M n-formyl-methionyl-leucyl-phenylanine (FMLP), a synthetic chemoattractant, enhanced subsequent superoxide release stimulated by phagocytosis. Van Epps and Garcia² observed that chemoattractants enhanced phagocytically stimulated superoxide release, phorbol myristate acetate (PMA) induced chemiluminescence, and neutrophil bactericidal activity. Issekutz et al.⁴ also reported that preincubation of neutrophils with chemoattractants markedly amplified the cells’ subsequent bactericidal potential.

The present report demonstrates that the responsiveness of the human neutrophil superoxide-generating system to PMA as well as to sodium fluoride (F⁻) is markedly enhanced by pretreatment of the cells with certain chemotactic factors. To extend these findings, we investigated the influence of chemoattractants on both the activity of the superoxide-generating enzyme and on the rate of enzyme activation. Our observations indicate that chemoattractants potentially influence both the activity and activation of the enzyme, and do so through separate and independently controlled cellular mechanisms.

Materials and Methods

Ferricytochrome-c, FMLP, Ficoll-Hypaque (Histopaque), ammonium chloride, zymosan, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co., St. Louis, Mo.; phorbol myristate acetate was from Consolidated Midland, Brewster, N.Y.; sodium fluoride, casein, dextrose, and sodium chloride were from Fisher Scientific, Pittsburgh, Pa.; Hanks’ balanced salt solution (BSS, no phenol red, Ca^{2+} = 1.0 mM; glucose = 1 mg/ml) was from Gibco, Grand Island, N.Y.; and beef lung heparin was from Upjohn, Kalamazoo, Mich. Superoxide dismutase was prepared from beef liver using the method of McCord and Fridovich.⁵

PMA and FMLP were dissolved in DMSO at a concentration of 10 mg/ml. They were further diluted in phosphate-buffered saline (PBS, pH 7.4) prior to use. Casein was dissolved in alkaline BSS at a concentration of 5 mg/ml and subsequently diluted in BSS, pH 7.4.

Polymorphonuclear leukocytes (neutrophils) were prepared from heparinized human blood using Ficoll-Hypaque density gradient centrifugation followed by ammonium chloride lysis of erythrocytes, as previously described.⁶ Experiments reported here have been repeated using cells obtained by a single step density gradient in which neither lysis nor enhanced sedimentation is required,⁷ and the results have been the same. Neutrophils were suspended at a concentration of 1.5 x 10^6/ml, unless otherwise indicated.

Serum was prepared from fresh blood by centrifugation. Zymosan-activated serum (ZAS) and casein-activated serum (CAS) were prepared by incubating fresh serum with 10 mg/ml of zymosan or casein at 37°C for 30 min.

The superoxide released from stimulated neutrophils was recorded by continuous assay using a system similar to that established by Cohen and Chovanec as modified by Newburger et al.⁸ Glass cuvettes (1 cm x 1 cm) contained, at 37°C, 1.5 x 10^6 neutrophils, 0.2 mM ferricytochrome-c, and additional reagents as

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indicated in a volume of 1.20 ml. The buffer used was BSS, unless otherwise indicated, and the temperature was maintained at 37°C. After equilibration for 5 min or after preincubation with chemotactic compounds for 5 min under the stated set of conditions, 50 μl of PMA or F- were added to achieve a final concentration of 100 ng/ml or 20 mM respectively. Reduction of cytochrome-c by O2- was continuously recorded at 550 nm using a Gilson 550 single beam spectrophotometer (slit width, 0.1 mm). The time (sec) after stimulation at which perceptible cytochrome-c reduction commenced was taken as the activation time, and the rate of reduction (after activation) taken as the activity of the O2- -generating system\(^a\) (see Fig. 1). That cytochrome-c was being reduced by O2- was evidenced by a greater than 98% inhibition of the response when 50 μg/ml of superoxide dismutase was added. The rate of superoxide release by neutrophils stimulated with either F- or PMA was initially linear (see below), thus permitting quantitation of enzyme activity (change in absorbance/min x 1000). In some cases, direct copies of the spectrophotometric recordings are presented. All reported experiments were repeated at least 4 times for verification, using neutrophils isolated from blood of separate individuals.

Neutrophil hexose monophosphate shunt (HMPS) activity stimulated by PMA was determined using methods similar to those described by Repine et al.\(^b\) and by Weening.\(^c\) Neutrophils (3 x 10\(^7\)/ml BSS) were incubated with 25 μCi 1\(^{14}\)C-glucose (New England Nuclear, Boston Mass.) for 2 hr at room temperature. One-half milliliter of the cell suspension was added to 2.5 ml of BSS in a 20 ml liquid scintillation counting vial that contained a separate inner vial filled with 0.8 ml hydroxide of Hyamine. In some experiments, leukocytes were preincubated for 5 min with 2 x 10\(^{-3}\) M FMLP after calibration with labeled glucose and prior to addition to the scintillation vials. Chemotactic peptide preincubated cells and cells not preexposed to chemotactic peptide were then stimulated with PMA (100 ng/ml) or FMLP (2 x 10\(^{-8}\) M). The scintillation vials were then sealed and incubated at 37°C for 20 min. Metabolic reactions were terminated by placing the vials in melting ice. Vials were left on ice for 4 hr to allow absorption of liberated \(^{14}\)CO\(_2\) by Hyamine hydroxide.\(^d\) The Hyamine was then dissolved in 10 ml of scintillation fluid (PPO-POPOP-toulene) and counted in a liquid scintillation counter. Results are expressed as mean counts per minute (cpm)/1.5 x 10\(^6\) neutrophils/20 min. Determinations were made in triplicate using neutrophils from a single individual. A second experiment with neutrophils from a different donor gave qualitatively similar results.

RESULTS

Figure 1 is a tracing of the spectrophotometric recording of cytochrome-c reduction by F- -stimulated neutrophils. The experiment shows the effect of preincubation with FMLP on subsequent oxidative responsiveness to F-. The chemotactic peptide enhanced the cells' capacity to generate O2- by exerting an influence on both the activation (time from stimulation to O2- release) and the activity of the F- -stimulated O2- -generating system. The concentrations of FMLP were lower than those necessary to effectively activate the O2- -generating enzyme, but were chemotactic in the presence of albumin. Chemotactic activity of FMLP was optimal at approximately 10\(^{-8}\) M, while >1.0 x 10\(^{-7}\) M FMLP was required for stimulation of oxidative metabolism.

Preincubation of neutrophils with low concentrations of ZAS also rendered them more responsive to F-. Both activation and activity of the F- -stimulated O2- -generating system were enhanced by ZAS in a dose-dependent manner (Fig. 2). Different preparations of ZAS held variable enhancing activity with respect to the amount necessary to cause an effect.

![Fig. 1. Chemotactic peptide enhancement of the activity and activation of human neutrophil O2- -generating system in response to 20 mM NaF. Tracing illustrates absorbance changes at 550 nm of solutions containing 1.5 x 10\(^6\) neutrophils and 0.2 mM cytochrome-c in BSS at 37°C. FMLP was added to two of the cuvettes 5 min prior to NaF, which was added at time 0. Effect of FMLP on activation time of the O2- -generating system is evidenced by a decrease in the lag (time from addition of F to initial increase in absorbance) of the upper two tracings. The rate of O2- -generation after activation (RATE) is expressed as the change in absorbance/min x 1000.](image-url)
The amount required for optimal enhancement ranged from 15 to 80 μl/ml. Solutions of ZAS of >2.5% were chemotactic. No attempt was made to correlate the amount of ZAS required for enhancement with the chemotactic potency of individual preparations.

While complement-derived chemoattractants and FMLP at relatively high concentrations stimulate leukocyte O2 generation,12,13 the chemoattractant casein and normal serum do not.14 Previous reports have also indicated that CAS holds at least as much chemotactic activity for human neutrophils as ZAS.15,16 Further, while low (chemotactic) concentrations of FMLP and ZAS desensitize the neutrophils’ ability to generate O2 upon exposure to 2.5 x 10⁻³ M FMLP, chemotactic concentrations of CAS, casein, and normal serum do not.14 It was of interest, therefore, to determine the influence of casein, CAS, and normal serum on the responsiveness of the superoxide-generating system stimulated by F⁻. Although each enhanced the rate of superoxide release (Table 1), the effect was less than that observed with FMLP or ZAS. Unlike FMLP and ZAS, casein, CAS, and normal serum did not alter the activation time of the O2-generating system in response to stimulation by F⁻.

At concentrations lower than those necessary to initiate oxidative metabolism, FMLP induces pronounced alterations in the membrane flux of calcium,17 a cation necessary for fluoride activation of the neutrophil O2-generating enzyme.18 We therefore investigated the role of Ca²⁺ in enhancement. As previously noted,18 cells prepared in Ca²⁺-free PBS generated very little O2 upon stimulation with 20 mM F⁻. If cells in Ca²⁺-free media were pretreated with 10⁻⁸ M FMLP, considerable O2 generation occurred upon stimulation with F⁻ (Fig. 3). The activation time of these cells was only slightly shorter than that of cells not exposed to FMLP but suspended...
in PBS containing Ca\(^{++}\) (0.5 mM). These results indicate that the influence of FMLP on activation time of the \(\mathbf{O}_2\) \(^{-}\)-generating system in response to \(\mathbf{F}^-\) was most pronounced when extracellular Ca\(^{++}\) was present. When Ca\(^{++}\) (0.5 mM) was added to neutrophils suspended in PBS with FMLP, the activation time of the \(\mathbf{O}_2\) \(^{-}\)-generating system stimulated by \(\mathbf{F}^-\) was further shortened (Fig. 3). The activation time for these cells was similar to the activation time of cells exposed to FMLP in BSS (Fig. 1). The rate of \(\mathbf{O}_2\) \(^{-}\) release from FMLP preexposed cells in response to \(\mathbf{F}^-\) was slower for cells in PBS without Ca\(^{++}\) than for those in PBS with 0.5 mM Ca\(^{++}\). However, it was not possible to determine whether this was due to a failure of enhancement in Ca\(^{++}\)-free media or to a failure of \(\mathbf{F}^-\) to optimally activate the \(\mathbf{O}_2\) \(^{-}\)-generating enzyme in the absence of extracellular Ca\(^{++}\). The rate for cells in PBS containing Ca\(^{++}\) was slightly less than that of cells in BSS. The activity of cells in PBS was not increased by adding glucose (1.0 mg/ml) prior to FMLP (data not shown).

Activation of neutrophil \(\mathbf{O}_2\) \(^{-}\)-generating system by PMA does not require extracellular Ca\(^{++}\), a fact we thought would permit investigation of the influence of extracellular Ca\(^{++}\) on chemotactic enhancement of the activity of the \(\mathbf{O}_2\) \(^{-}\)-generating system under conditions where omission of the cation would not compromise activation of the system. Similar to the results obtained with \(\mathbf{F}^-\), neutrophils treated with FMLP in BSS were markedly activated in their capacity to generate \(\mathbf{O}_2\) \(^{-}\)-free media or to a failure of \(\mathbf{F}^-\) to optimally activate the \(\mathbf{O}_2\) \(^{-}\)-generating enzyme in the absence of extracellular Ca\(^{++}\). The rate for cells in PBS containing Ca\(^{++}\) was slightly less than that of cells in BSS. The activity of cells in PBS was not increased by adding glucose (1.0 mg/ml) prior to FMLP (data not shown).

To address the possibility that the effect of FMLP on the \(\mathbf{O}_2\) \(^{-}\)-generating system stimulated by PMA was merely a result of increased \(\mathbf{O}_2\) \(^{-}\) release rather than increased production, neutrophil oxidative metabolism, as reflected by HMPS activity, was determined. At a concentration of \(2 \times 10^{-8} \text{ M}\), FMLP caused very little release of \(\mathbf{1^4CO}_2\) from \(1\text{-}\mathbf{1^4C}\)-glucose as compared to values obtained with unstimulated neutrophils (unstimulated neutrophils, 1174 ± 84 cpm; FMLP-stimulated neutrophils 1371 ± 37 cpm). Neutrophils stimulated with 100 ng/ml PMA released 6551 ± 340 cpm of \(\mathbf{1^4CO}_2\). When neutrophils that had been preincubated with \(2 \times 10^{-8} \text{ M}\) FMLP were stimulated with 100 ng/ml of PMA, a striking enhancement of oxidative metabolism initiated by PMA was noted. Chemotactic factor pretreated cells released 20,281 ± 1100 cpm of \(\mathbf{1^4CO}_2\). A similar result was noted when FMLP and PMA were added to the neutrophils simultaneously (20,253 ± 1138 cpm). It thus appears that the effect of chemoattractants on neutrophil superoxide release is associated with an increased capacity of the cells to generate \(\mathbf{O}_2\) \(^{-}\) from NADPH.

**DISCUSSION**

The continuous assay of neutrophil \(\mathbf{O}_2\) \(^{-}\) release of Cohen and Chovaniec is a valuable method for the simultaneous determination of the rate of activation and the activity of the superoxide-generating system. This method has been used to identify processes in the pathway from stimulation to \(\mathbf{O}_2\) \(^{-}\) release. That the stimulation of \(\mathbf{O}_2\) \(^{-}\) release by different agents may be mediated by different mechanisms is suggested by the observation that extracellular Ca\(^{++}\) is required for the oxidative activity induced by \(\mathbf{F}^-\), but not for that in PBS containing Ca\(^{++}\) (0.5 mM). These results indicate that the influence of FMLP on activation time of the \(\mathbf{O}_2\) \(^{-}\)-generating system in response to \(\mathbf{F}^-\) was most pronounced when extracellular Ca\(^{++}\) was present. When Ca\(^{++}\) (0.5 mM) was added to neutrophils suspended in PBS with FMLP, the activation time of the \(\mathbf{O}_2\) \(^{-}\)-generating system stimulated by \(\mathbf{F}^-\) was further shortened (Fig. 3). The activation time for these cells was similar to the activation time of cells exposed to FMLP in BSS (Fig. 1). The rate of \(\mathbf{O}_2\) \(^{-}\) release from FMLP preexposed cells in response to \(\mathbf{F}^-\) was slower for cells in PBS without Ca\(^{++}\) than for those in PBS with 0.5 mM Ca\(^{++}\). However, it was not possible to determine whether this was due to a failure of enhancement in Ca\(^{++}\)-free media or to a failure of \(\mathbf{F}^-\) to optimally activate the \(\mathbf{O}_2\) \(^{-}\)-generating enzyme in the absence of extracellular Ca\(^{++}\). The rate for cells in PBS containing Ca\(^{++}\) was slightly less than that of cells in BSS. The activity of cells in PBS was not increased by adding glucose (1.0 mg/ml) prior to FMLP (data not shown).

Table 2. Requirement of Extracellular Glucose for FMLP Enhancement of the Rate of Neutrophil \(\mathbf{O}_2\) \(^{-}\) Release Stimulated by PMA

<table>
<thead>
<tr>
<th>Media*</th>
<th>Preincubation†</th>
<th>Activation Time‡ (sec)</th>
<th>Activity§ (Δ A/min × 1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS</td>
<td>PBS</td>
<td>108 (102-114)</td>
<td>68 (50-85)</td>
</tr>
<tr>
<td></td>
<td>FMLP†</td>
<td>54 (42-66)</td>
<td>141 (135-145)</td>
</tr>
<tr>
<td>PBS</td>
<td>PBS</td>
<td>111 (102-126)</td>
<td>59 (50-70)</td>
</tr>
<tr>
<td></td>
<td>FMLP†</td>
<td>66 (54-78)</td>
<td>57 (40-75)</td>
</tr>
<tr>
<td>PBS + glucose</td>
<td></td>
<td>126 (114-138)</td>
<td>63 (55-70)</td>
</tr>
<tr>
<td></td>
<td>FMLP</td>
<td>51 (42-54)</td>
<td>143 (135-150)</td>
</tr>
</tbody>
</table>

*Neutrophils were prepared in PBS at a concentration of 1.5 x 10^7/ml; 100 μ of neutrophils were added cuvettes containing 0.95 ml of BSS, PBS, or PBS + glucose.
†50 μ of PBS or FMLP in PBS were added to the cuvettes containing neutrophils in the indicated buffer and incubated for 5 min at 37°C prior to the addition of 100 ng/ml PMA.
‡Time from addition of PMA to initiation of \(\mathbf{O}_2\) \(^{-}\) release; mean and (range) of 4 determinations.
§Rate of \(\mathbf{O}_2\) \(^{-}\) release, average and range of 4 determinations.
||The glucose concentration was 1.26 mg/ml.
†Final concentration, 2 x 10^-8 M.
induced by PMA. The two systems need not be vastly different. As indicated by the data of this report, the absolute requirement of extracellular Ca for activation of the O2-generating system stimulated by F- can be eliminated by pretreatment of neutrophils with FMLP.

Our study confirms previous observations that neutrophils exposed to chemotactic factors are enhanced in their responsiveness to oxidative stimulation by nonchemotactic agents. By continuously assaying O2 release, we have extended these findings to demonstrate an effect of the chemoattractants FMLP and ZAS on both the activity of the O2-generating system and on its rate of activation. Our data suggest that the mechanism by which chemoattractants enhance the response triggered by F- is not the same as that which results in enhanced responses to PMA. Thus, while enhancement of activity stimulated by PMA required extracellular glucose (but not Ca++), extracellular glucose was not required for enhancement of activity stimulated by F-. Whereas chemotactic factor enhancement of activation in response to F- was dependent on the presence of extracellular Ca++, chemotactic enhancement of activation in response to PMA was not. As many as four separate cellular mechanisms activated by chemotactic factors may result in enhanced oxidative responsiveness. These include a process dependent on extracellular Ca++ for enhanced activation of the F-stimulated system, a glucose-dependent process for enhanced activity of the PMA-stimulated system, and extracellular Ca++ and glucose independent processes for enhanced activity and activation of the F- and PMA-stimulated systems, respectively. The finding that chemotactic concentrations of casein, CAS, and normal serum enhanced the activity but not the activation of the O2-generating system stimulated by F- can be taken as a further indication that separate and independently controlled processes underlie enhancement by chemoattractants of the activation and the activity of the neutrophil O2-generating system.

The results of the experiments reported in Table 2 were obtained with 100 ng/ml PMA, a concentration that does not maximally activate neutrophil oxidative metabolism. In preliminary experiments, we observed, in confirmation of the results of Newburger et al., that 1 μg/ml PMA elicited maximal O2 release as reflected by both activation time and activity. We further observed that neutrophils preincubated with 2 × 10^-8 M FMLP displayed an enhanced response to 1 μg/ml of PMA. The results were qualitatively similar to those reported in Table 2; chemotactic factor activated cells responded to 1 μg/ml of PMA with a shorter activation time and enhanced rate of O2 release in comparison to cells not previously exposed to chemotactic factor. We chose the lower concentration of PMA to quantitate and characterize the phenomenon because the slower kinetics of the responses allowed more accurate evaluation of the spectrophotometric recordings.

In summary, these data support the hypothesis that neutrophils at sites of infection and inflammation are activated as a result of previous encounters with chemoattractants. The active state is characterized by an increased responsiveness of cells to stimuli of oxidative activation. Cells respond faster and with greater vigor upon stimulation. Investigation of the mechanism of enhancement demonstrates that at least two separate mechanisms can be involved, one dependent on extracellular glucose and one on Ca++. The role of chemotactic enhancement of oxidative metabolism in host defense remains to be established.

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