Stimulation and Priming of Human Neutrophils by Interleukin-8: Cooperation With Tumor Necrosis Factor and Colony-Stimulating Factors

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Interleukin-8 (IL-8) stimulated an increase in cytoplasmic-free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and intracellular pH (pH\(_i\)) in parallel at low concentrations (0.5 to 5 ng/mL), and stimulated O\(_{2}^{-}\) release and membrane depolarization in parallel at high concentrations (50 to 5,000 ng/mL). IL-8-induced O\(_{2}^{-}\) release was potentiated by tumor necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte-CSF (G-CSF) in a dose-dependent manner, whereas it was inhibited by cyclic AMP agonists. These characteristics and the time-courses of the responses stimulated by IL-8 were similar to those stimulated by N-formyl-methionyl-leucyl-phenylalanine (FMLP), except that the cells stimulated by IL-8 showed shorter duration and less magnitude in some responses. In addition, IL-8 was found to be a potent priming agent and to enhance O\(_{2}^{-}\) release stimulated by FMLP.

INTERLEUKIN-8 (IL-8), a 8-kD protein expressed by platelets, monocytes, and certain granulocytes, is a chemotactic factor for neutrophils, eosinophils, and monocytes, and a chemoattractant for lymphocytes in vitro. IL-8 is also a potent activator of neutrophils. Thus, we studied the effects of IL-8, TNF, and GM-CSF on human neutrophils and compared these with the effects of FMLP.

We and others have recently reported that cytokines such as TNF, GM-CSF, and G-CSF prime human neutrophils and enhance superoxide (O\(_{2}^{-}\)) release stimulated by a chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP). However, little is known about the interaction between IL-8 and FMLP, and there is no report regarding the cooperative effects of IL-8 and other cytokines, including TNF, GM-CSF, and G-CSF, on human neutrophil functions. In this report, we show that IL-8 stimulates or primes human neutrophils according to its concentrations and cross-talks with TNF, GM-CSF, G-CSF, or FMLP at the inflammatory sites.

MATERIALS AND METHODS

Reagents. Highly purified (> 98%) recombinant human IL-8 (72 amino acid type, an apparent molecular weight of 8,359) was provided by Dainippon Pharmaceutical Co, Ltd (Osaka, Japan). Highly purified recombinant human GM-CSF and G-CSF produced by Escherichia coli were provided by Schering-Plough Co, Ltd (Osaka, Japan) and Conray-Ficoll (Mallinckrodt Inc, St Louis, MO), respectively.

Preparation of cells. Neutrophils were prepared from healthy adult donors as described, using dextran sedimentation, centrifugation with Conray-Ficoll, and hypotonic lysis of contaminated erythrocytes. Neutrophil fractions were suspended in Hank's balanced salt solution (HBSS) and contained greater than 95% neutrophils.

Determination of O\(_{2}^{-}\) release. O\(_{2}^{-}\) was assayed by superoxide dismutase-inhibitable reduction of ferricytochrome C, and the continuous assay was performed in a Hitachi 557 spectrophotometer (a double-wavelength spectrophotometer; Hitachi Ltd, Tokyo, Japan) equipped with thermostatted cuvette holder (37°C) as described. Final cell concentration was 1 × 10\(^6\) cells/mL. The priming effect of IL-8 was very rapid and was maximal within 5 minutes of preincubation. The dose-response curves for priming were identical to those for triggering of an increase in [Ca\(^{2+}\)]\(_i\) and pH\(_i\). The potency of the maximal priming effects on FMLP-induced O\(_{2}^{-}\) release was TNF > GM-CSF > IL-8 > G-CSF. The combination of IL-8 and the suboptimal concentrations of TNF or GM-CSF resulted in the additive priming effect, whereas the combination of the optimal concentrations of IL-8 and the optimal concentration of TNF, GM-CSF, or G-CSF resulted in the effect of more potent priming agent alone. These findings suggest that IL-8 stimulates or primes human neutrophils according to its concentrations and cross-talks with TNF, GM-CSF, G-CSF, or FMLP at the inflammatory sites.

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reduction of cytochrome C was measured at 550 nm with a reference wavelength at 540 nm, and the time course of cytochrome C reduction (the absorbance change at 550 to 540 nm) was followed on the recorder. The amount of O$_2^-$ release was calculated from cytochrome C reduced for 5 minutes after the addition of stimuli.

**Determination of membrane potential changes.** Changes in the transmembrane potential were measured by using di-O-C$_3$(3) as described. The fluorescence was measured with a Hitachi MPF-4 fluorescence spectrophotometer (Hitachi Ltd) equipped with a thermostatted cuvette holder (37°C). The final concentration of di-O-C$_3$(3) was 0.25 μmol/L, and the final cell concentration was 1 x 10$^6$ cells/mL. The excitation and emission wavelength were set at 460 and 510 nm, respectively. The magnitude of membrane potential changes was calculated from the maximal change after the addition of stimuli and was expressed as the percentage of the resting level.

**Determination of cytoplasmic-free Ca$^{2+}$.** Cytoplasmic-free Ca$^{2+}$ ([Ca$^{2+}$]) was measured by using quin 2 as described. The fluorescence was measured with a Hitachi MPF-4 fluorescence spectrophotometer equipped with a thermostatted cuvette holder (37°C). The final cell concentration was 2.5 x 10$^6$ cells/mL. The excitation and emission wavelength were set at 339 and 492 nm, respectively.

**Determination of intracellular pH.** Intracellular pH (pH$_i$) was measured by using BCECF as described. Cells (4 x 10$^6$/mL) suspended in HEPES buffer (153 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L glucose, 20 mmol/L HEPES, pH 7.4) were prewarmed at 37°C for 5 minutes. BCECF acetoxymethylester (3 μmol/L) was added and the cells were incubated at 37°C for 30 minutes in a shaking water bath. After loading, the cells were washed twice and suspended in HEPES buffer containing 1 mmol/L CaCl$_2$. The fluorescence was measured with a Hitachi F-4010 fluorescence spectrophotometer (Hitachi Ltd) equipped with a thermostatted cuvette holder (37°C). The final cell concentration was 3 x 10$^6$ cells/mL. The excitation and emission wavelength were set at 500 and 530 nm, respectively.

**Statistical analysis.** Student's t-test was used to determine statistical significance.

**RESULTS**

**Interrelationships between O$_2^-$ release, membrane potential changes, an increase in [Ca$^{2+}$], and changes in pH$_i$ in human neutrophils stimulated by IL-8.** As shown in Fig 1, human neutrophils stimulated by IL-8 showed transient release of O$_2^-$, the changes in transmembrane potential characterized by initial depolarization followed by partial repolarization, transient increase in [Ca$^{2+}$], and the changes in pH$_i$ characterized by initial acidification followed by sustained alkalization. The dose-response curves for triggering of O$_2^-$ release and membrane depolarization by IL-8 were almost identical to each other, and almost maximal stimulation of both responses was obtained at 10$^{-7}$ mol/L. On the other hand, much lower concentrations of FMLP were sufficient to stimulate an increase in [Ca$^{2+}$], and an increase in pH$_i$ at lower concentrations of FMLP were observed at 10$^{-7}$ mol/L, and almost maximal increases in [Ca$^{2+}$] and pH$_i$ were observed at 10$^{-5}$ mol/L of FMLP; the concentration that induced little changes in transmembrane potential and no release of O$_2^-$ (Fig 3). The initial acidification was not observed at lower concentrations of FMLP (<10$^{-6}$ mol/L). As shown in Table 1, IL-8-induced O$_2^-$ release was inhibited by dibutyryl cAMP and PGE, in a dose-dependent manner. FMLP-induced O$_2^-$ release was also inhibited by cAMP agonists (data not shown). Thus, the characteristics of these responses triggered by IL-8 and FMLP were fundamentally similar to each other. Effects of TNF, GM-CSF, and G-CSF on O$_2^-$ release in...
human neutrophils stimulated by IL-8. It has been recently shown that TNF, GM-CSF, and G-CSF prime human neutrophils and enhance $O_2^-$ release stimulated by receptor-mediated $Ca^{2+}$-mobilizing agonists such as FMLP (Table 2). Upon our recent studies have shown that maximal priming is obtained by the pretreatment of neutrophils with 33 ng/mL (100 U/mL) TNF, 5 ng/mL GM-CSF, or 50 ng/mL G-CSF for 10 minutes at 37°C. As shown in Fig 4, when human neutrophils were primed by these cytokines under the optimal conditions, $O_2^-$ release stimulated by IL-8 was significantly enhanced. The enhancing effects of these cytokines were TNF > GM-CSF > G-CSF. Although the enhancing effect of G-CSF was minimal (approximately 1.3-fold), significant enhancement ($P < .05$) was consistently observed when 50 ng/mL of G-CSF was used. The enhancing effects of TNF and GM-CSF were dependent on the concentrations of these cytokines used (Fig 5). The enhancing effects of TNF and GM-CSF on neutrophil $O_2^-$ release were observed over the complete range of the effective concentrations of IL-8 (Fig 5). The dose-response curves for triggering of $O_2^-$ release by IL-8 in TNF-primed, GM-CSF-primed, or control cells were identical and were not shifted to the left in primed cells. Thus, low concentrations of IL-8 ($<5$ ng/mL) did not trigger detectable release of $O_2^-$ even in the neutrophils primed optimally by TNF or GM-CSF (Fig 5).

Effect of IL-8 on $O_2^-$ release in human neutrophils stimulated by FMLP. IL-8 not only triggered $O_2^-$ release in neutrophils but also primed neutrophils for enhanced release of $O_2^-$. As shown in Fig 6, FMLP-induced $O_2^-$ release was markedly enhanced by the pretreatment of cells with IL-8 for 5 minutes at 37°C. Significant priming ($P < .01$) was observed at $0.5$ ng/mL (60 pmol/L), and almost maximal priming was obtained at $5$ ng/mL (600 pmol/L) of IL-8 (Figs 6 and 7). The dose-response curves for priming the cells were almost identical to those for triggering of an increase in $[Ca^{2+}]$, and an increase in pH (Figs 2 and 7). The priming of cells by IL-8 was dependent on the preincubation time, and the preincubation of cells with IL-8 (5 ng/mL) for 5 minutes at 37°C was sufficient to obtain the maximal effect (Fig 7).

Combined effects of IL-8 and TNF, GM-CSF, or G-CSF on $O_2^-$ release in human neutrophils stimulated by FMLP. As shown in Table 2, the potency of the maximal priming effect...
Dibutyryl cAMP or PGE, for 5 minutes at 37°C and then stimulated with IL-8 and TNF, GM-CSF, or G-CSF for 10 minutes at 37°C. When human neutrophils were preincubated with the optimal concentration of IL-8 (5 ng/mL) and the suboptimal concentration of TNF (0.3 ng/mL) or GM-CSF (0.2 ng/mL), the additive priming effect was obtained (Table 2). Similar results were obtained when lower concentrations (<5 ng/mL) of IL-8 and the suboptimal concentrations of TNF or GM-CSF were used (data not shown). On the other hand, when the optimal concentrations of IL-8 (5 ng/mL) and the optimal concentration of TNF (33 ng/mL), GM-CSF (5 ng/mL), or G-CSF (50 ng/mL) were used, neither synergistic nor additive effects were obtained and the priming effect of more potent cytokine alone was observed (Table 2).

**Table 2. Combined Effects of IL-8 and G-CSF, GM-CSF, or TNF on O₂⁻ Release in Human Neutrophils Stimulated by FMLP**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>O₂⁻ Release (nmol O₂⁻/min)</th>
<th>Stimulated by FMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.57 ± 0.46</td>
<td>7.97 ± 0.86*</td>
</tr>
<tr>
<td>G-CSF (50 ng/mL)</td>
<td>5.26 ± 0.84*</td>
<td>8.01 ± 0.28*</td>
</tr>
<tr>
<td>GM-CSF (0.2 ng/mL)</td>
<td>6.28 ± 0.88*</td>
<td>11.93 ± 1.43*†</td>
</tr>
<tr>
<td>GM-CSF (6 ng/mL)</td>
<td>12.49 ± 0.90†</td>
<td>11.99 ± 0.104</td>
</tr>
<tr>
<td>TNF (0.3 ng/mL)</td>
<td>7.13 ± 1.41†</td>
<td>12.73 ± 1.21*‡</td>
</tr>
<tr>
<td>TNF (33 ng/mL)</td>
<td>20.49 ± 3.32‡</td>
<td>22.99 ± 5.68†</td>
</tr>
</tbody>
</table>

Cells (1 x 10⁶/mL) were preincubated with the indicated concentrations of G-CSF, GM-CSF, or TNF in the absence or presence of IL-8 (5 ng/mL) for 10 minutes at 37°C before IL-8 release was measured. The data are expressed as means ± SD of three to six experiments.

**Discussion**

The ability of IL-8 to trigger the respiratory burst in human neutrophils is controversial. Several studies have reported that IL-8 triggered release of O₂⁻ and H₂O₂ in human neutrophils and the amount of O₂⁻ release triggered by IL-8 was almost comparable with that triggered by FMLP. On the other hand, Djeu et al reported that IL-8 did not trigger O₂⁻ release. In the present experiments, we showed that IL-8 at high concentrations did trigger O₂⁻ release in human neutrophils in a dose-dependent manner, although its potency was much lower than that of FMLP. It is unlikely that these different observations are attributable to the difference in sources or preparations of IL-8, because three independent groups have reported that various preparations of IL-8, including natural IL-8 produced by human monocytes and recombinant IL-8 produced by E. coli or mammalian cells, have identical biologic activities. In the present experiments, we used recombinant IL-8 produced by E. coli, the same preparation of IL-8 as that used by Djeu et al. The low potency of IL-8 to trigger the respiratory burst might explain the failure of detection of O₂⁻ release by the end-point assay or the double-beam spectrophotometer that Djeu et al used. We used a double-wavelength spectrophotometer for the continuous assay of O₂⁻ release, which excludes the possible interruption of the assay by the turbidity in the reaction mixtures and can detect the small change of cytochrome C reduction. Neutrophils used by several previous studies were prepared from blood stored for up to 20 hours at 4°C to 10°C, which might explain the higher amount of O₂⁻ release stimulated by IL-8, because the O₂⁻-releasing capacity appears to increase during the aging of neutrophils in vitro.

IL-8 stimulated an increase in [Ca²⁺], and an increase in pH, in parallel at low concentrations, and stimulated O₂⁻ release and membrane depolarization in parallel at high concentrations. IL-8-induced O₂⁻ release was potentiated by TNF, GM-CSF, or G-CSF, and was inhibited by cAMP agonists. These characteristics and the time-courses of the responses stimulated by IL-8 were similar to those stimulated by FMLP, except that the cells stimulated by IL-8...
The present experiments show that higher concentrations of IL-8 (50 to 5,000 ng/mL, 6 to 600 nmol/L) was required to stimulate $O_2^-$ release and membrane depolarization. Lindley et al. reported that dose-dependent stimulation of H$_2$O$_2$ release determined by chemiluminescence was observed at 3 to 30 nmol/L of natural and recombinant IL-8. It has been also reported that higher concentrations of IL-8 (100 to 1,000 nmol/L) was required to stimulate the maximal release of elastase, maximal surface expression of adhesion receptors (CD11b/CD18), and maximal adherence to biologic surfaces. On the other hand, much lower concentrations of IL-8 (0.5 to 5 ng/mL, 60 to 600 pmol/L) were sufficient to induce increases in [Ca$^{2+}$], and pH$_i$ in human neutrophils. Essentially similar findings were observed in neutrophils stimulated by FMLP. These findings suggest that increases in [Ca$^{2+}$], and pH$_i$ are not a sufficient signal to induce respiratory burst, membrane depolarization, exocytosis, and adhesion in human neutrophils, and that additional signals produced by higher occupation of the receptors are required for the maximal stimulation of these functions. In any case, all these responses could be provoked at the inflammatory sites, where sufficient amount of IL-8 would be accumulated. In these situations, IL-8-induced $O_2^-$ release may contribute to the tissue damage, which could be potentiated by TNF, GM-CSF, or G-CSF.

IL-8 at lower concentrations (0.5 to 5 ng/mL, 60 to 600 pmol/L) primed neutrophils and enhanced $O_2^-$ release stimulated by FMLP. The dose-response curves for priming are identical to those for inducing neutrophil chemotaxis and for triggering increases in [Ca$^{2+}$], and pH$_i$ (Figs 2 and 7). In addition, recent studies showed that the concentrations of IL-8 detected in synovial fluids from patients with rheumatoid arthritis and nonrheumatoid arthritis were 0.83 to 8.80 ng/mL, the concentrations sufficient for inducing neutrophil chemotaxis and for maximal priming of cells. These findings taken together suggest that the physiologic role of IL-8 is to induce neutrophil showed shorter duration and less magnitude in some responses. In addition, interrelationships between $O_2^-$ release, membrane potential changes, an increase in [Ca$^{2+}$], and changes in pH$_i$ in neutrophils stimulated by IL-8 were essentially similar to those stimulated by FMLP. All these findings together suggest that IL-8 and FMLP stimulate neutrophils, at least in part, through similar mechanisms, and support the contention that the chemotactic factors, including IL-8 and FMLP, may share the common signaling pathways.
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Fig 7. Effect of IL-8 on O₂⁻ release in human neutrophils stimulated by FMLP. The data are expressed as means ± SD of three experiments. (Top) Cells (1 x 10⁶/mL) were preincubated with indicated concentrations of IL-8 for 5 minutes at 37°C before FMLP (10⁻⁷ mol/L) was added. (Bottom) Cells (1 x 10⁶/mL) were preincubated with IL-8 (5 ng/mL) for indicated periods at 37°C before FMLP (10⁻⁷ mol/L) was added.

chemotaxis and to prime neutrophils during migration to the inflammatory sites. The studies with dose-response curves indicate that an increase in [Ca²⁺]ᵢ, an increase in pHᵢ, or both may be associated with the priming of cells by IL-8. IL-8-induced increase in [Ca²⁺]ᵢ was transient and returned to the resting level within 3 minutes, whereas the optimal priming was observed at 5 minutes. These findings suggest that the metabolic events provoked by an increase in [Ca²⁺]ᵢ, rather than an increased level of [Ca²⁺]ᵢ itself are responsible for the priming. Another possibility is that a sustained increase in pHᵢ in neutrophils stimulated by IL-8 may be, at least in part, responsible for increased release of O₂⁻ stimulated by later addition of FMLP, because it has been reported that FMLP-induced O₂⁻ release in human neutrophils is modulated by pHᵢ and enhanced by an increased level of pHᵢ (intracellular alkalinization). In this regard, it is of interest that the combination of IL-8 and the suboptimal concentration of TNF or GM-CSF, both of which were also detected in synovial fluids from patients with rheumatoid arthritis, resulted in the additive priming effects, whereas the combination of the optimal concentration of IL-8 and the optimal concentration of TNF, GM-CSF, or G-CSF resulted in the effects of more potent cytokine alone. The similar findings were observed in the combination of GM-CSF and G-CSF. These findings suggest that the common mechanisms for priming are shared by IL-8, TNF, GM-CSF, and G-CSF. As shown in the present study, IL-8 induced a sustained increase in pHᵢ and a transient increase in [Ca²⁺]ᵢ. On the other hand, GM-CSF is reported to induce an increase in pHᵢ but not an increase in [Ca²⁺]ᵢ. We observed that TNF and G-CSF also induced an increase in pHᵢ (unpublished observations, January 1991), but not an increase in [Ca²⁺]ᵢ. These findings taken together suggest that an increased level of pHᵢ induced by IL-8, TNF, GM-CSF, or G-CSF may be a common mechanism for enhanced release of O₂⁻ stimulated by FMLP, although it is possible that other mechanisms may also work in priming by these cytokines. In addition, these findings indicate that the presence of multiple cytokines at the inflammatory sites does not necessarily cause excessive priming of neutrophils. It is conceivable that these phenomena may contribute to prevent excessive tissue damage by activated neutrophils.

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