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Diverse Effects of Cytochalasin B on Priming and Triggering the Respiratory Burst Activity in Human Neutrophils and Monocytes

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Abstract

Cytochalasin B, despite its potent enhancing effect on superoxide (O_2^-) release triggered by N-formyl-methionyl-leucyl-phenylalanine (FMLP) and many other agonists, significantly inhibited O_2^- release triggered by interleukin 8 (IL-8) and platelet-activating factor in human neutrophils. Cytochalasin B also enhanced changes in membrane potential stimulated by FMLP but inhibited those stimulated by IL-8. Using IL-8 as a triggering agonist, we found that the priming effect of tumor necrosis factor (TNF) and granulocyte-macrophage colony–stimulating factor (GM-CSF) on O_2^- release was slightly but significantly potentiated by cytochalasin B. O_2^- release triggered by TNF and GM-CSF was completely abolished by cytochalasin B. In contrast to these diverse effects of cytochalasin B on O_2^- release, changes in cytoplasmic pH stimulated by FMLP, IL-8, TNF, and GM-CSF were not or were only minimally affected by cytochalasin B. Unlike human neutrophils, human monocytes stimulated by FMLP showed inhibition of O_2^- release and changes in membrane potential in response to cytochalasin B, and the priming effect of TNF and GM-CSF on O_2^- release in human monocytes was completely abolished by cytochalasin B. These findings indicate the diverse effects of cytochalasin B on phagocytes and suggest distinct regulatory mechanisms according to the functions, agonists, and cell types. *Int J Hematol.* 2001;74:409-415.

Key words: Cytochalasin B; Human neutrophils; Human monocytes; Respiratory burst

1. Introduction

Cytochalasin B is a fungal metabolite that has been thought to inhibit actin polymerization and disrupt microfilaments in cells [1]. In addition to these well-known effects on the cytoskeleton, cytochalasin B has conventionally been used as an agent that potentiates agonist-induced responses, including respiratory burst and exocytosis in neutrophils [2-8]. Furthermore, several groups of investigators consistently add cytochalasin B to reaction mixtures in experiments on respiratory burst and exocytosis to more efficiently detect agonist-induced superoxide (O_2^-) release and degranulation [2-7]. In spite of, or, alternatively, because of such widespread use of this agent, the effects of cytochalasin B on signaling pathways in human neutrophils are not fully understood, and little is known about its effects on human monocytes or macrophages.

Recent studies have clarified that cytokines such as tumor necrosis factor (TNF), granulocyte-macrophage colony–stimulating factor (GM-CSF), and G-CSF are potent activators of the respiratory burst system in human neutrophils [9-14]. The stimulatory effects of these cytokines on the respiratory burst in neutrophils are essentially classified into 2 categories, priming effects and triggering effects. TNF, GM-CSF, and G-CSF rapidly (within 10 minutes) prime neutrophils for enhanced release of O_2^- stimulated by a chemotactic peptide [9-11,13,14], and TNF and GM-CSF trigger O_2^- release directly over a prolonged course (for up to 120 minutes) in suspended neutrophils [10-12,14]. These marked differences in the kinetics of priming and triggering of the respiratory burst stimulated by cytokines strongly suggest different mechanisms for priming and triggering. Our preliminary observations showed that O_2^- release triggered by TNF and GM-CSF

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was markedly inhibited by cytochalasin B (K.S., M.Y., S.K., A.Y., unpublished data, 2001), suggesting involvement of a cellular target of the cytochalasin B in cytokine-triggered respiratory burst, although the possible involvement of cytochalasin B in priming has never been studied. Thus, one of the important aims of this study is to compare and clarify the mechanisms of priming and triggering using cytochalasin B.

In addition to these cytokines, another family of chemotactic cytokines, chemokines, has been identified and intensively studied by investigators of phagocytes [15,16]. We have also reported the effects of interleukin 8 (IL-8), a chemokine, on O_2^- release and signaling pathways in human neutrophils [17]. Findings of our study and observations by others clearly demonstrated that the biological effects of IL-8 are essentially identical to those of the bacterial chemoattractant N-formyl-methionyl-leucyl-phenylalanine (FMLP) [15,17], ie, both IL-8 and FMLP induced O_2^- release and changes in membrane potential and cytoplasmic pH (pHi) in a very similar manner [17]. These findings are reasonable, based on the knowledge that receptors for IL-8 and FMLP share the same structure, with high amino acid homology, and are both classified as G-protein–coupled receptors with 7 transmembrane domains [15,16,18]. There is, however, no information regarding the possible differences in the action of these 2 chemotactic factors, which have extremely different structures and origins.

In this study, we used cytochalasin B as a tool to explore mechanisms of the activating system for respiratory burst in human phagocytes stimulated by receptor-mediated agonists, such as cytokines, chemokines, and chemotactic peptides. Unexpectedly, we found that O_2^- release triggered by FMLP and IL-8 is entirely different in its sensitivity to cytochalasin B. These findings were further confirmed and extended by studying changes in membrane potential, which are known to be closely associated with the respiratory burst in phagocytes [4,10,14]. On the other hand, cytochalasin B exerted no or minimal effects on agonist-induced changes in pHi. We also demonstrated that priming and triggering of the respiratory burst in human neutrophils and monocytes are both regulated by cytochalasin B–sensitive, but distinct, mechanisms.

2. Materials and Methods

2.1. Reagents

Highly purified recombinant human G-CSF, GM-CSF, TNF, and IL-8 produced by *Escherichia coli* were kindly provided by Kirin Brewery Co, Ltd (Tokyo, Japan); Schering Plough Co, Ltd (Osaka, Japan); Dainippon Pharmaceutical Co, Ltd (Osaka); and Dainippon Pharmaceutical Co, Ltd (Osaka), respectively. Contamination of lipopolysaccharide was less than 100 pg/mg protein. Cytochrome C, superoxide dismutase, and FMLP were purchased from Sigma Chemical Co (St. Louis, MO, USA); platelet-activating factor (PAF) was from Bachem (Bubendorf, Switzerland); 2′,7′-bis(carboxyethyl)-5(6)- carboxyfluorescein (BCECF) acetoxymethylester was from Dojindo Laboratories (Kumamoto, Japan); 3,3′-dipentyloxacarbocyanine (di-O-C_5[3]) was from the Japanese Research Institute for Photosensitizing Dyes (Okayama, Japan); Conray was from Mallinckrodt, Inc (St. Louis, MO, USA); and Ficoll was from Pharmacia Fine Chemicals, Inc (Piscataway, NJ, USA).

2.2. Preparation of Cells

Human neutrophils and mononuclear cells were prepared from heparinized venous blood samples from healthy adult donors as described [14,19], using dextran sedimentation, centrifugation with Conray-Ficoll, and hypotonic lysis of contaminated erythrocytes. Monocytes were further purified from mononuclear cells by centrifugal elutriation in a Hitachi SR6Y elutriation rotor (Hitachi Ltd, Tokyo, Japan) as described [19]. Neutrophil fractions contained >95% neutrophils, and monocyte fractions contained 85% to 95% monocytes and 5% to 15% lymphocytes. Both fractions were suspended in Hanks' balanced salt solution (HBSS). After informed consent was obtained, human alveolar macrophages were obtained by bronchoscopic sterile lavage of the left or right lung of patients with lung cancer without infection. Mononuclear cells were separated from the lavage fluid by centrifugation with Conray-Ficoll and suspended in HBSS. Mononuclear fractions used in these experiments contained 80% to 95% alveolar macrophages and 5% to 20% lymphocytes.

2.3. Determination of O_2^- Release

O_2^- was assayed spectrophotometrically by superoxide dismutase–inhibitable reduction of ferricytochrome C, and a continuous assay was performed using a Hitachi 557 spectrophotometer (double-wavelength spectrophotometer; Hitachi Ltd, Tokyo, Japan) equipped with a thermostat cuvette holder (37°C) as previously described [14,19]. The cell suspension in HBSS was added to a 1 mL cuvette containing 110 μM ferricytochrome C to a final volume of 0.990 to 0.9975 mL. The final cell concentration was 1×10^6 cells/mL. The reaction mixture in the cuvette was prewarmed for 3 minutes at 37°C. The cuvette was put in a thermostat cuvette holder (37°C) of the spectrophotometer and the reduction of cytochrome C was measured at 550 nm, with a reference wavelength at 540 nm. Various stimulating agents (2.5-10 μL) were added to the reaction mixture in cuvettes to a final volume of 1 mL and the desired concentration of these agents, and the time course of cytochrome C reduction (the absorbance change at 550-540 nm) was followed on a recorder. The amount of O_2^- release was calculated from cytochrome C reduced 5 minutes after the addition of a stimulus. In some experiments, O_2^- release was determined by the endpoint assay as described [14].

2.4. Determination of Changes in Membrane Potential

Changes in the transmembrane potential were measured using di-O-C_5(3) as described [13]. The fluorescence was measured using a Hitachi F-4010 fluorescence spec-

trophotometer equipped with a thermostat cuvette holder (37°C). The cell suspension in HBSS was added to a 3-mL cuvette containing 25 μM di-O-C_5(3) to a final volume of 3 mL. The final cell concentration was 1×10^6 cells/mL. The cells were equilibrated with dye for 10 minutes at 37°C before a stimulus was added. The cell suspension was maintained by means of a magnetic flea and stirrer. The excitation and emission wavelengths were set at 460 and 510 nm, respectively. The magnitude of the changes was calculated from the maximal change after the addition of a stimulus and was expressed as a percentage of the resting level.

2.5. Determination of pHi

pHi was measured using BCECF as described [13]. Cells (4×10^7/mL) suspended in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer (153 mM NaCl, 5 mM KCl, 5 mM glucose, 20 mM HEPES, pH 7.4) were prewarmed at 37°C for 5 minutes. BCECF acetoxymethylester (3 μM) was added, and the cells were incubated for 30 minutes at 37°C in a shaking water bath. After loading, the cells were washed twice and suspended in HEPES buffer containing 1 mM $CaCl_2$. The fluorescence was measured with a Hitachi F-4010 fluorescence spectrophotometer equipped with a thermostat cuvette holder (37°C). The final cell concentration was 3×10^6 cells/mL. The excitation and emission wavelengths were set at 500 and 530 nm, respectively.

2.6. Statistical Analysis

Student *t* test was used to determine significance.

3. Results

3.1. Effects of Cytochalasin B on O_2^- Release and Changes in Membrane Potential in Human Neutrophils Stimulated by FMLP, IL-8, and PAF

It is well known that cytochalasin B enhances O_2^- release in neutrophils stimulated by various agonists, including chemotactic peptides, plant lectins, and Ca^{2+} ionophores [2-8]. We confirmed this finding using FMLP as an agonist (Figure 1). In addition, cytochalasin B also enhanced changes in membrane potential (membrane depolarization) stimulated by FMLP (Figure 1), a finding consistent with previous observations showing a close association between O_2^- release and changes in membrane potential in human neutrophils [4,10,14]. In marked contrast to these findings for FMLP, cytochalasin B slightly but significantly inhibited O_2^- release and membrane depolarization stimulated by IL-8 (65% ± 6% [n = 4] of control for O_2^- release and 66% ± 3% [n = 3] of control for membrane depolarization) (Figure 1). To clarify whether this phenomenon was specific for and limited to IL-8–induced responses, we studied the effects of cytochalasin B on O_2^- release stimulated by another chemotactic factor, platelet-activating factor (PAF). PAF (10^{-6} M) by itself triggered a transient release of O_2^- along a similar

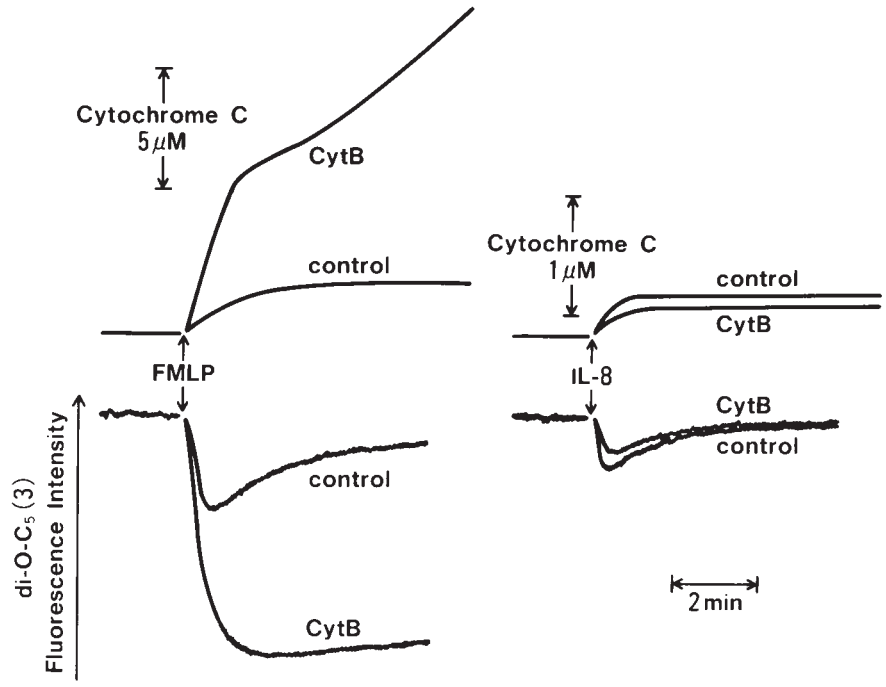

Figure 1. Serial changes in O_2^- release and membrane potential in human neutrophils stimulated by N-formyl-methionyl-leucyl-phenylalanine (FMLP) or interleukin 8 (IL-8). Cells (1×10^6/mL) were preincubated with or without cytochalasin B (CytB) (5 μg/mL) for 2 minutes at 37°C and were then stimulated with FMLP (10^{-7} M) and IL-8 (500 ng/mL).

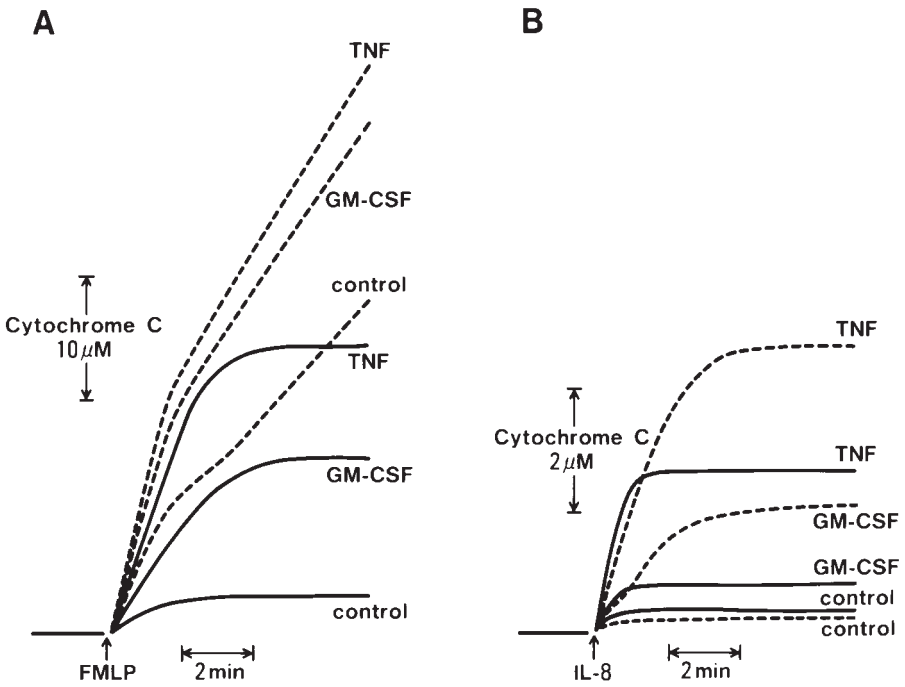

Figure 2. Serial changes in O_2^- release and membrane potential in human neutrophils stimulated by N-formyl-methionyl-leucyl-phenylalanine (FMLP) (A) or interleukin 8 (IL-8) (B). Cells (1×10^6/mL) were preincubated with granulocyte-macrophage colony–stimulating factor (GM-CSF) (5 ng/mL) and tumor necrosis factor (TNF) (50 ng/mL) in the absence (solid line) or presence (dotted line) of cytochalasin B (5 μg/mL) for 10 minutes at 37°C and were then stimulated with FMLP (10^{-7} M) and IL-8 (500 ng/mL).

time course to IL-8 in human neutrophils (0.43 ± 0.07 nmol/5 min per 1×10^6 cells). Cytochalasin B did not enhance but instead inhibited PAF-induced O_2^- release in human neutrophils by 44% ± 10% (n = 3). Cytochalasin B by itself neither triggered O_2^- release nor provoked membrane-potential changes in human neutrophils (data not shown).

3.2. Effects of Cytochalasin B on Priming and Triggering of O_2^- Release in Human Neutrophils Stimulated by GM-CSF and TNF

To clarify the effects of cytochalasin B on priming of the respiratory burst in neutrophils, we studied the priming effects of cytokines on agonist-induced O_2^- release in the absence and presence of cytochalasin B. In the presence of cytochalasin B (5 μg/mL), maximal priming effects of the optimal concentrations of TNF (50 ng/mL) and GM-CSF on FMLP-induced O_2^- release in human neutrophils were still detectable (Figure 2). It is, however, difficult to evaluate the net effect of cytochalasin B on priming itself, because of the potent enhancing effect of cytochalasin B on FMLP-induced responses in human neutrophils. To avoid this problem, we used IL-8 instead of FMLP as a triggering agonist of O_2^- release, because the effect of cytochalasin B on O_2^- release stimulated by IL-8 was inhibitory and not marked (Figure 1). As shown in Figure 2B, maximal priming effects of TNF and GM-CSF on IL-8–induced O_2^- release in human neutrophils were not impaired but instead were augmented in the presence of 5 μg/mL cytochalasin B.

In contrast to these findings for priming, O_2^- release triggered directly by optimal concentrations of TNF (50 ng/mL) or GM-CSF (5 ng/mL) was almost completely inhibited by the concomitant addition of cytochalasin B (5 μg/mL) (5% ± 1% [n = 3] and 9% ± 3% [n = 4] of control in TNF- and GM-CSF-stimulated cells, respectively). O_2^- release triggered by TNF or GM-CSF was also inhibited when cytochalasin B was added to the reaction mixture after the onset of O_2^- release (10 minutes after the addition of TNF or GM-CSF) (data not shown).

3.3. Effect of Cytochalasin B on the Changes in pHi in Human Neutrophils Stimulated by FMLP and IL-8, and TNF and GM-CSF

Changes in pHi have been reported to play an important role in the functional activation of human neutrophils [13,17,20]. To further explore the effects of cytochalasin B on the activation system of human neutrophils, we investigated the effect of cytochalasin B on agonist-induced changes in pHi in human neutrophils. Both FMLP and IL-8 induced transient acidification followed by sustained alkalinization in human neutrophils [17], whereas TNF and GM-CSF induced sustained alkalinization after 2 to 5 minutes of lag time, during which no change in pHi was observed [13]. Cytochalasin B alone induced initial acidification followed by sustained alkalinization in human neutrophils, although at a much lower magnitude than that induced by other agonists such as FMLP and IL-8 (time courses not shown). Cytochalasin B,

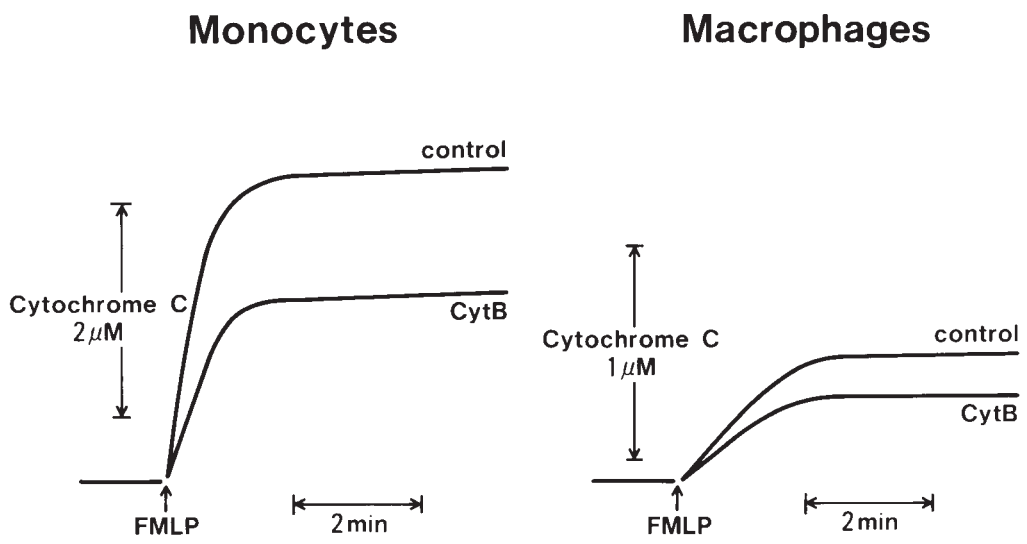

Figure 3. Serial changes in O_2^- release in human monocytes and macrophages. Human peripheral blood monocytes (1×10^6 cells/mL) (left panel) and human alveolar macrophages (1×10^6 cells/mL) (right panel) were preincubated with or without cytochalasin B (CytB) (5 μg/mL) for 2 minutes at 37°C and were then stimulated with N-formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-6} M).

however, had no fundamental effect on intracellular acidification and alkalinization induced by FMLP and IL-8, although the initial acidification induced by FMLP and IL-8 appeared to be slightly dampened by cytochalasin B because of the intracellular alkalinizing effect of cytochalasin B itself. In addition, intracellular alkalinization induced by TNF and GM-CSF was also unaffected by cytochalasin B (data not shown). Thus, the regulatory system of pHi does not contain a cytochalasin B–sensitive component in human neutrophils.

3.4. Effects of Cytochalasin B on O_2^- Release and Changes in Membrane Potential in Human Monocytes and on O_2^- Release in Human Macrophages

As previously reported, human monocytes, such as neutrophils, release O_2^- in response to FMLP [19]. In marked contrast to the effect of cytochalasin B in human neutrophils, FMLP-induced O_2^- release in human monocytes was not enhanced but instead was slightly and significantly inhibited by cytochalasin B (Figure 3 and Table 1). This finding is a specific inhibitory phenomenon, because O_2^- release in human monocytes stimulated by concanavalin A was markedly enhanced by cytochalasin B (data not shown). Membrane-potential changes (membrane depolarization) in human monocytes stimulated by FMLP were also inhibited by cytochalasin B (75% ± 6% [n = 3] of control). Using FMLP as a triggering agonist of O_2^- release, we studied the effect of cytochalasin B on priming in human monocytes. In marked contrast to the priming effect in human neutrophils, the priming effect of TNF and GM-CSF on FMLP-induced O_2^- release in human monocytes was completely abolished in the presence of cytochalasin B (Table 1).

To further explore the effect of cytochalasin B on the respiratory burst of normal human monocyte/macrophage lineages, we investigated the effect of cytochalasin B on O_2^- release in human alveolar macrophages. Stimulation of human alveolar macrophages with FMLP provoked transient release of O_2^-, without a detectable lag time (Figure 3). The pattern of the time course was almost identical to that of FMLP-induced O_2^- release in human neutrophils and monocytes, although the amount of O_2^- released appeared to be less in macrophages. Pretreatment of human macrophages with cytochalasin B did not enhance but instead inhibited FMLP-induced O_2^- release by 62% ± 5% (n = 3).

4. Discussion

It has been generally accepted that the respiratory burst in neutrophils is potentiated by the actions of cytochalasin B, because O_2^- release in neutrophils stimulated by various unrelated agonists, including chemotactic factors (FMLP and the other chemotactic peptides), plant lectins (concanavalin

Table 1.
Effects of Cytochalasin B on Priming and Triggering of O_2^- Release in Human Monocytes*

	O_2^- Release, nmol/5 min per 1×10^6 cells	
Priming Agonists	Cytochalasin B Absent	Cytochalasin B Present
Control	2.88 ± 0.17	2.13 ± 0.20
IL-3	4.04 ± 0.27	2.06 ± 0.06
M-CSF	4.10 ± 0.15	2.02 ± 0.06
GM-CSF	5.22 ± 0.42	1.97 ± 0.24
TNF	6.15 ± 0.82	1.96 ± 0.18

*Cells (1×10^6/mL) were preincubated with interleukin 3 (IL-3) (50 ng/mL), macrophage colony-stimulating factor (M-CSF) (100 ng/mL), granulocyte-macrophage CSF (GM-CSF) (5 ng/mL), and tumor necrosis factor (TNF) (50 ng/mL) in the absence or presence of cytochalasin B (5 μg/mL) for 10 minutes at 37°C and were then stimulated with N-formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-6} M) for release of O_2^-. The data are expressed as mean ±SD of 3 to 5 experiments.

A and wheat germ agglutinin), and Ca^{2+} ionophores (ionomycin and A23187), is enhanced by pretreatment of cells with cytochalasin B and by its delayed addition [2-5,21]. These previous findings strongly suggest the involvement of a cellular target of cytochalasin B, such as microfilaments, in the activating system of the respiratory burst. On the other hand, this study clarified that O_2^- release in human neutrophils stimulated by IL-8 and PAF was not enhanced but instead was inhibited by cytochalasin B. In addition, the gradual release of O_2^- stimulated by TNF and GM-CSF was almost completely abolished by cytochalasin B. These findings further confirm the essential and pivotal roles of the cellular target of cytochalasin B in the respiratory burst and provide novel insight into its distinct stimulus-related contributions.

It has been repeatedly observed that chemotactic factors such as FMLP and IL-8 activate neutrophils via common mechanisms, and the effects of these chemotactic factors on signaling pathways are, although quantitatively different, qualitatively identical [15-17]. In addition, receptors for FMLP, IL-8, and PAF share substantial amino acid sequence homology and a common structure [18]. The findings here, however, showed an apparent difference in the actions of these chemotactic factors, FMLP versus IL-8 and PAF, in their responsiveness to cytochalasin B. To clarify the reason for the marked differences between FMLP and IL-8 (or PAF), we investigated the effects of cytochalasin B on signaling pathways and on changes in membrane potential and in pHi in human neutrophils stimulated by FMLP and IL-8. The findings are as follows: (*a*) cytochalasin B markedly enhanced the membrane depolarization stimulated by FMLP but inhibited that stimulated by IL-8; (*b*) cytochalasin B had no effects on changes in pHi stimulated by FMLP or IL-8. These findings suggest that cytochalasin B acts on neutrophils via a common upstream component shared by the O_2^--producing system and the membrane-potential change–provoking system, but not shared by the pHi-regulating system. In addition, the cytochalasin B–sensitive component participates in the signaling pathways of these 2 chemotactic factors via extremely different mechanisms; that is, the signaling element following the G-protein–coupled receptors for FMLP is distinct from that for IL-8 in relation to the respiratory burst and changes in membrane potential.

Using IL-8 as a stimulus, we found that the priming effects of TNF and GM-CSF on O_2^- release in human neutrophils were not inhibited but instead were slightly enhanced by cytochalasin B, suggesting a relatively minor contribution of the cellular target of cytochalasin B in priming. The nature of the cytochalasin B–resistant mechanism of priming remains to be determined. On the other hand, O_2^- release triggered by TNF or GM-CSF was almost completely inhibited by cytochalasin B. All these findings together suggest that TNF and GM-CSF prime or trigger the respiratory burst in neutrophils via distinct and independent mechanisms. Marked differences in the time course of priming and triggering by TNF and GM-CSF (priming reached peak levels within 10 minutes [9-11,13], whereas triggering continued for 60-120 minutes [9,11,14]) also support this idea.

Changes in pHi stimulated by cytokines and chemotactic factors were not affected by cytochalasin B. In particular, intracellular alkalinization, which is known to be mediated by an Na^+/H^+ exchanger [22], was resistant to the action of cytochalasin B irrespective of the agonists used, and, thus, neither the Na^+/H^+ exchanger itself nor its activating system use cytochalasin B–sensitive pathways. These findings for intracellular alkalinization, particularly by TNF and GM-CSF, may partly explain the mechanisms of priming by these cytokines, because intracellular alkalinization alone can enhance O_2^- production [20]. Additionally, both intracellular alkalinization and priming of the respiratory burst were resistant to cytochalasin B.

In marked contrast to the findings for human neutrophils, the findings for human monocytes and macrophages show that O_2^- release and changes in membrane potential in human monocytes and O_2^- release in human macrophages stimulated by FMLP were not enhanced but instead were inhibited by cytochalasin B. In addition, the priming effect of TNF and GM-CSF on FMLP-induced O_2^- release in monocytes was completely abolished by cytochalasin B. Although the regulation of the respiratory burst in human neutrophils and monocytes has several common characteristics [19,21], our findings clearly show that the mechanisms of respiratory burst activation in human monocytes are, at least in some aspects, entirely different from those in human neutrophils. Specifically, opposite effects of cytochalasin B on the priming of neutrophils and monocytes clearly indicate mechanistic differences in the priming of the respiratory burst among different types of mature human phagocytes. One possible explanation for this phenomenon is that actin polymerization, which is known to be inhibited by cytochalasin B [1,23], plays a key role in priming human monocytes but not human neutrophils. On the other hand, intracellular alkalinization, which was resistant to the action of cytochalasin B, might play an important role in priming neutrophils but not monocytes, because cytokine-induced intracellular alkalinization was observed only in human neutrophils but not (or only minimally) in human monocytes [13,24]. Interestingly, direct triggering of the respiratory burst, which was observed in human neutrophils stimulated by TNF and/or GM-CSF [10-12,14], was highly sensitive to the action of cytochalasin B but was not observed in human monocytes [24].

Findings of the current investigation demonstrate the diverse effects of cytochalasin B on the respiratory burst and signaling pathways in mature human phagocytes stimulated by physiological receptor-mediated agonists such as chemotactic factors and cytokines. These findings indicate that each receptor-mediated agonist stimulates various metabolic events in phagocytes such as neutrophils or monocytes via agonist-specific and cytochalasin B–sensitive or –insensitive pathways. These findings have not been reported for the immature progenitor cells of phagocytes and hematopoietic cells of other lineages, and they show agonist-specific and cell-type–specific mechanisms for activating mature leukocyte effector functions.

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