Interleukin-8 (IL-8), Melanoma Growth-stimulatory Activity, and Neutrophil-activating Peptide Selectively Mediate Priming of the Neutrophil NADPH Oxidase through the Type A or Type B IL-8 Receptor*

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The capacity of neutrophils to generate superoxide (O$_2^-$) can be enhanced by prior exposure to “priming” agents such as interleukin-8 (IL-8), melanoma growth-stimulatory activity (MGSA), and neutrophil-activating peptide (ENA-78). The biological effects of these chemokines are mediated by at least two distinct receptors: type A (IL-8-RA) and type B (IL-8-RB). Using neutralizing monoclonal antibodies to IL-8-RA and IL-8-RB, we have investigated the contribution each receptor makes to the priming response. Preincubation with IL-8, MGSA, or ENA-78 enhanced the ability of neutrophils to generate O$_2^-$ following stimulation with the bacterial peptide formyl-Met-Leu-Phe. The priming effect of IL-8 was eliminated by an anti-IL-8 monoclonal antibody (mAb) that is known to bind IL-8 with high affinity and prevent receptor occupancy. Incubation of neutrophils with a neutralizing mAb specific for IL-8-RA blocked IL-8-induced priming but had no effect on priming by MGSA or ENA-78. In contrast, treatment with a neutralizing mAb specific for IL-8-RB failed to inhibit the priming effect of IL-8 but blocked both MGSA and ENA-78-induced priming. These observations indicate that the priming effect of IL-8 on the neutrophil respiratory burst is predominantly mediated via IL-8-RA, whereas priming by MGSA and ENA-78 is mediated by IL-8-RB.

One of the primary roles of neutrophils is to provide the body with an efficient defense mechanism against microorganisms. The generation of superoxide anion radicals (O$_2^-$) by a process known as the respiratory burst, is an essential component of the neutrophil’s microbicidal armory. The NADPH oxidase, responsible for the respiratory burst, is a complex, multicomponent electron transport enzyme that transfers electrons from NADPH to molecular oxygen to form superoxide. The terminal electron donor in this chain is cytochrome b$_{558}$, which consists of two subunits, gp91phox and p22phox. Three other components that are critical for enzymatic activity are the cytosolic components, p47phox, p67phox, and the Rho-related GTP-binding protein Rac (1, 3, 4).

Activation or “triggering” of the neutrophil respiratory burst by agents such as phorbol myristate acetate or the bacterial peptide formyl-Met-Leu-Phe (FMLP) results in the generation of large amounts of O$_2^-$ (1). The capacity of neutrophils to generate O$_2^-$ can be augmented by prior exposure to a variety of “priming” agents including cytokines, growth factors, cell-permeant lipids, ionophores, and chemokines (5). Primed cells do not actively generate superoxide but respond to a subsequent triggering agent with a greatly enhanced respiratory burst (5). The biological functions, receptor types, and signaling pathways for these priming agents are widely different, suggesting that priming of the neutrophil NADPH oxidase may commonly occur in vivo.

Interleukin-8 (IL-8) and melanoma-growth-stimulatory activity (MGSA) are proinflammatory agents that prime the neutrophil respiratory burst (6–8). The chemokines, IL-8, MGSA, and neutrophil-activating peptide (ENA-78), play a key role in the recruitment and activation of neutrophils in vivo (9–14). Interleukin-8 and MGSA are secreted by a wide variety of cell types in response to inflammatory stimuli (12), whereas ENA-78 is produced predominantly by epithelial cells and fibroblasts (15). These three molecules belong to the CXC chemokine family, which is characterized by having two conserved cysteine residues separated by one amino acid in the NH$_2$-terminal region (10). Exposure of neutrophils to IL-8, MGSA, or ENA-78 induces a wide range of biological responses including an intracellular calcium flux, changes in intracellular pH, chemotaxis, cytoskeleton reorganization, and shape change (6, 14–21). These responses are likely to be required to attract neutrophils to the site of inflammation and activate them for efficient microbial killing.

The biological effects of IL-8 are mediated by two seven-transmembrane G-protein-coupled receptors: type A (IL-8-RA) and type B (IL-8-RB). Both receptors bind IL-8 with high affinity (1–2 nM); however, in contrast to IL-8-RA, IL-8-RB also binds other CXC chemokines including MGSA, ENA-78, and neutrophil-activating peptide-2 with high affinity (14, 15, 22). The IL-8 receptors show 32% homology in the N-terminal extracellular region, almost identical transmembrane domains, and an overall amino acid homology of 77% (23). In this report we have characterized the ability of IL-8, MGSA, and ENA-78 to prime the neutrophil respiratory burst and the capacity of an anti-IL-8 mAb to neutralize the priming effect of IL-8. In addition, using mAbs that specifically bind to the IL-8 type A or B receptors and prevent ligand binding, we have determined the contribution each receptor makes to the priming response.

EXPERIMENTAL PROCEDURES

Reagents—The following were obtained commercially: horse heart cytochrome c type III, N-formyl-Met-Leu-Phe, and superoxide dis-
mutase from Sigma; goat anti-mouse IgG fluorescein isothiocyanate, mouse IgG1, and IgG2a from Becton Dickinson; and Ficoll-Paque from Pharmacia Biotech, Sweden. End-X™ B15 endotoxin removal affinity resin was from Associates of Cape Cod, Inc. Recombinant human IL-8 and MGSA were generously supplied by W. Darbonne and Dr. Henry Lowman, respectively (Genentech, Inc.). Recombinant human ENA-78 and MGSA were generously supplied by W. Darbonne and Dr. Henry Lowman, respectively (Genentech, Inc.). Recombinant human IL-8 was from R & D Systems, Inc.

Preparation of Human Neutrophils—Human neutrophils were isolated from whole blood obtained by venipuncture and purified by dextran sedimentation, hypotonic cell lysis to remove residual erythrocytes, and density gradient centrifugation (24). Cells were resuspended in phosphate-buffered saline (PBS) at 2 × 10^6/ml for 30 min at 4°C. After washing, cells were resuspended in PBS containing 1.0% fetal bovine serum and incubated with monoclonal antibodies (1 μg/ml) for 30 min at 4°C. After washing, cells were resuspended in PBS, 1.0% fetal bovine serum containing 50 μg/ml fluorescein isothiocyanate-conjugated goat anti mouse IgG and incubated for 30 min at 4°C. Cells were washed twice in PBS, 1.0% fetal bovine serum, read on a FACSscan (Becton Dickinson), and analyzed using Cell-Quest software.

Generation of mAbs—Monoclonal antibodies against IL-8-RA (9H1) and IL-8-RB (10H2) were generated by immunizing BALB/c mice with transfected 293 cells as described previously (26, 27). Monoclonal antibody against recombinant human IL-8 (A5.12.14) was generated by immunizing mice with an IL-8-ubiquitin fusion protein.

RESULTS

Incubation of human neutrophils with 1 nM IL-8, MGSA, or ENA-78 at 37°C rapidly enhanced the ability of FMLP to trigger a respiratory burst (Fig. 1). The ability of these chemokines to prime the FMLP-triggered respiratory burst was evident after a 10-min incubation period at 37°C, and maximal priming occurred between 10 and 15 min. The priming effect of all three chemokines was stable for at least 30 min (Fig. 1).

Treatment of neutrophils with 0–1 μM IL-8, MGSA, or ENA-78 alone failed to activate O$_2^-$ generation, indicating that these chemokines do not directly modulate the activity of the NADPH oxidase (Fig. 2). The priming effect of the three chemokines was dose-dependent, with maximal priming occurring between 1 and 10 nM (Fig. 2). Incubation of neutrophils with 1 nM IL-8, MGSA, and ENA-78 for 10 min at 37°C enhanced the FMLP-triggered respiratory burst from 14 ± 2 (unprimed) to 41 ± 5, 33 ± 7, and 33 ± 3 nmol/min/10^7 cells, respectively (Fig. 2), indicating that these chemokines mediate priming via a high affinity interaction with IL-8 receptors.

The priming effect of IL-8 was inhibited by an anti-IL-8 mAb, A5.12.14 (Fig. 3). This mAb binds to IL-8 with high affinity (K$_d$ = 0.3 nM) and prevents the interaction of IL-8 with both IL-8-RA and IL-8-RB. Pretreatment of IL-8 (1 nM) with A5.12.14 (5 and 10 nM) for 20 min at 22°C blocked the priming effect of IL-8 by 89 ± 2% and 99 ± 7%, respectively (Fig. 3). This anti-IL-8 mAb has also been shown to inhibit IL-8-mediated

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chemotaxis and elastase release in human neutrophils.\textsuperscript{3} The ability of this anti-IL-8 mAb to block neutrophil priming indicates that under our experimental conditions, priming was exclusively mediated by IL-8 and not by contaminating buffer reagents, such as endotoxin.

We have recently generated mAbs, 9H1 and 10H2, that specifically recognize IL-8-RA and IL-8-RB on the surface of human neutrophils (Fig. 4) and stably transfected 293 cells expressing IL-8-RA or IL-8-RB (26, 27). Both 9H1 and 10H2 are known to block the binding of IL-8 to IL-8-RA and IL-8-RB, respectively (26, 27). Incubation of neutrophils with 10–1600 nM 9H1 or 10H2 for 10–30 min at 37 °C failed to prime or trigger the neutrophil respiratory burst (Fig. 5).\textsuperscript{4} Since these mAbs fail to prime or trigger the NADPH oxidase, we sought to determine whether 9H1 and 10H2 could block the priming effects of IL-8, MGSA, and ENA-78. Incubation of neutrophils with 9H1 but not 10H2 (0.6–660 nM) for 5 min at 37 °C prior to the addition of 1 nM IL-8 inhibited priming by up to 80 ± 14% (Fig. 6a). This inhibition was not observed using the isotype control mAbs IgG\textsubscript{1} and IgG\textsubscript{2a} (Fig. 6a). Incubation of neutrophils with a combination of 9H1 and 10H2 showed no further inhibitory activity on the IL-8-mediated priming response than that already caused by 9H1 alone (Fig. 6b). In contrast, incubation with 10H2 but not 9H1 (0.6–660 nM) for 5 min at 37 °C prior to the addition of 1 nM MGSA or ENA-78 blocked priming in a dose-dependent manner (Fig. 7). Treatment with 6.6 nM 10H2 blocked MGSA- and ENA-78-mediated priming by 97 ± 10% and 100 ± 10% respectively. These results suggest that IL-8 mediates priming predominantly via interaction with IL-8-RA, whereas MGSA and ENA-78 mediate priming exclusively via IL-8-RB.

**DISCUSSION**

Interleukin-8, MGSA, and ENA-78 exert a number of biological effects on circulating neutrophils including chemotaxis, cytoskeletal reorganization, shape change, and degranulation. We have confirmed previous investigations demonstrating that IL-8 (6–8, 28) and MGSA (8) prime the neutrophil NADPH oxidase and report that a related CXC chemokine, ENA-78, also exhibits this activity. As observed for other priming agents

\textsuperscript{3} C. A. Hébert, unpublished data.

\textsuperscript{4} S. P. Green, A. Chuntharapai, and J. T. Curnutte, unpublished data.
Importantly, incubation of neutrophils with these mAbs alone did not prime, trigger, or inhibit the FMLP-triggered respiratory burst, indicating that they do not directly modulate NADPH oxidase activity. The inhibitory effect of 9H1 on IL-8-mediated priming was not further potentiated by pretreatment with a combination of both 9H1 and 10H2. This result supports our observation that IL-8-induced priming occurs independently of IL-8-RB activation.

Melanoma growth-stimulatory activity and ENA-78 also rapidly primed the neutrophil NADPH oxidase. Similar to IL-8, priming with MGSA and ENA-78 was observed between 5 and 10 min at concentrations between 0.1 and 100 nM (Figs. 1 and 2). Since MGSA and ENA-78 bind IL-8-RB with high affinity ($K_d = 1–2$ nM) but IL-8-RA with low affinity ($K_d = 500$ nM) (15, 21, 22), priming by these chemokines is likely to be mediated by IL-8-RB. The ability of 10H2 to completely block the priming effect of MGSA and ENA-78 supports this notion (Fig. 7). Further, pretreatment of neutrophils with an anti-IL-8-RA mAb, 9H1, failed to inhibit the priming effects of MGSA and ENA-78. These data indicate that activation of IL-8-RA by engagement with MGSA or ENA-78 is necessary and sufficient for priming of the NADPH oxidase. In contrast, our data suggest that binding of IL-8 to IL-8-RB is insufficient to prime neutrophils.

Hammond et al. (17) and Quan et al. (37) have employed a similar approach using neutralizing anti-IL-8 receptor mAbs to investigate the role each receptor plays in mediating neutrophil chemotaxis. Neutrophil chemotaxis induced by MGSA was blocked by an anti-IL-8-RA mAb, whereas an anti-IL-8-RA mAb had no effect (17). In contrast, these investigators concluded that the majority of IL-8-induced chemotaxis was mediated by IL-8-RA (17, 37) and that only a minor proportion was mediated by IL-8-RB (17). These data, together with our observations, indicate that IL-8-RA may be predominantly responsible for mediating the biological responses of IL-8 on neutrophil chemotaxis and priming. These findings raise the possibility that other physiological functions elicited by IL-8 in human neutrophils, such as exocytosis, actin polymerization, and changes in intracellular pH, may be mediated principally by IL-8-RA.

The molecular mechanism(s) responsible for priming of the neutrophil respiratory burst by IL-8, MGSA, or ENA-78 are not understood. It has been suggested that IL-8 increases the percentage of cells that generate oxidants following exposure to FMLP (28, 33). This change may be related to an increase in cell surface expression or affinity of the FMLP receptors on neutrophils following exposure to the chemokine (8, 38). Alternatively, priming may alter $Ca^{2+}$, tyrosine kinase, or phospholipid-dependent signaling pathways, allowing a more efficient transduction of the stimulatory signals required for activation of the NADPH oxidase.

The observation that IL-8, MGSA, ENA-78, PAF, and C5a have direct agonist functions for chemotaxis, shape change, and actin polymerization (6, 14–21, 39) but have little (C5a, PAF) or no (IL-8, MGSA, ENA-78) effect on oxidase activation suggests the biochemical signals required for these functions
are not sufficient for stimulation of the neutrophil respiratory burst. Translocation of the cytosolic components of the NADPH oxidase to the plasma membrane is thought to be an important step for assembly of the oxidase and subsequent generation of superoxide (1, 2). In agreement with previous investigations (40–42), we have shown that activation of the oxidase with phorbol 12-myristate 13-acetate or FMLP results in the translocation of p47phox and 67phox; however, this activity was not observed following treatment with priming agents such as IL-8.5 The inability of IL-8 to cause translocation of cytosolic oxidase components may explain why chemokines are not agonists for NADPH oxidase.

IL-8-RA and IL-8-RB exhibit a high level of conservation in the first three intracellular loops (97.7%), which are known to be required for G-protein coupling and calcium mobilization (43). The cytoplasmic carboxyl tail, however, exhibits sequence diversity, and in this region the two receptors exhibit only 44% homology. This observation raises the possibility that the carboxyl tail of the IL-8 receptors may play a role in determining functional specificity. Using progressive deletion mutants of IL-8-RB, it has been postulated that the carboxyl tail amino acids between positions 317 and 324 play an important role in the functional effects of IL-8 on neutrophil priming. In human neutrophils, IL-8, but not PMA, activation of phospholipase D is specific to the type A receptor (48). Since IL-8-RB by engagement with MSGA and ENA-78 was sufficient to prime the neutrophil respiratory burst in the presence of an anti-IL-8-RA mAb, implies that engagement of IL-8 to IL-8-RB is not sufficient to prime the neutrophil NADPH oxidase. An understanding of the contribution each IL-8 receptor makes to neutrophil function is critical in elucidating the pathophysiology of neutrophil-mediated inflammatory diseases such as ischemic reperfusion injury and acute lung injury where IL-8 is likely to play a key role.

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