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Importance of MEK in Neutrophil Microbicidal Responsiveness

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Exposure of neutrophils to inflammatory stimuli such as the chemoattractant FMLP leads to activation of responses including cell motility, the oxidative burst, and secretion of proteolytic enzymes. A signaling cascade involving sequential activation of Raf-1, mitogen-activated protein kinase (MEK), and extracellular signal regulated kinase (ERK) is also rapidly activated after agonist exposure. The temporal relationship between these events suggests that the kinases may be involved in triggering the effector functions, but direct evidence of a causal relationship is lacking. To assess the role of the MEK/ERK pathway in the activation of neutrophil responses, we studied the effects of PD098059, a potent and selective inhibitor of MEK. Preincubation of human neutrophils with 50 μM PD098059 almost completely (>90%) inhibited the FMLP-induced activation of MEK-1 and MEK-2, the isoforms expressed by neutrophils. This dose of PD098059 virtually abrogated chemoattractant-induced tyrosine phosphorylation and activation of ERK-1 and ERK-2, implying that MEKs are the predominant upstream activators of these mitogen-activated protein kinases. Pretreatment of neutrophils with the MEK antagonist inhibited the oxidative burst substantially and phagocytosis only moderately. In addition, PD098059 antagonized the delay of apoptosis induced by exposure to granulocyte-macrophage CSF. However, the effects of PD098059 were selective, as it failed to inhibit other responses, including chemoattractant-induced exocytosis of primary and secondary granules, polymerization of F-actin, chemotaxis, or activation of phospholipase A2. We conclude that MEK and ERK contribute to the activation of the oxidative burst and phagocytosis, and participate in cytokine regulation of apoptosis.

using expression of either dominant negative or constitutively activated forms of MEK suggest that these enzymes constitute the principal route for ERK activation (18–21). Three isoforms of MEK, termed MEK-1 to -3 have been described (17, 22), and both MEK-1 and MEK-2, which phosphorylate and activate ERK, have been detected in neutrophils, where they are activated by bacterial chemotactic peptides (23, 24).

Some of the functions attributed to MAPKs, such as cytoskeletal remodeling (25) and activation of phospholipase A₂ (26), may be important to the microbial activity of neutrophils. Moreover, it has been suggested that MAPK may participate in the activation of the neutrophil oxidative burst. One of the cytosolic components of the NADPH oxidase, p47(phox), contains two serine residues within a sequence that is recognized by proline-directed kinases such as ERK (27, 28), and phosphorylation of p47(phox) is associated with oxidative activation (2). However, a direct link between the ERK pathway and activation of neutrophil effector functions, including the oxidative burst and granule secretion, has not been established.

The purpose of the current study was to establish the physiologic significance of activation of the MEK-MAPK pathway in several aspects of the complex microbialicidal response of neutrophils. To accomplish this goal, we employed the specific MEK inhibitor PD098059, developed previously in one of our laboratories (29). This compound blocks the activation (and phosphorylation) of MEK through an allosteric mechanism that does not involve inhibition of ATP binding (30). The potency and specificity of this inhibitor have been extensively documented in a variety of biologic systems, and, of potential importance for leukocytes, PD098059 does not block the activity of MKK3 and MKK4 (29, 30).

**Materials and Methods**

**Materials and media**

FMLP, TPA, EGTA, HEPES, ATP, GM-CSF, zymosan, PMSF, aprotinin, horseradish peroxidase, pepstatin A, leupeptin, ferricytochrome c, superoxide dismutase, and RPMI 1640 were obtained from Sigma Chemical Co. (St. Louis, MO). Albumin was obtained from Calbiochem (La Jolla, CA). Protein A-Sepharose, dextran T-500, and Ficoll–Paque were purchased from Pharmacia LKB Biotechnology, Inc. (Baie d'Urle, Quebec, Canada). Prestained molecular weights standards were purchased from Bio-Rad (Hercules, CA). Immobilon filters were obtained from Millipore (Bedford, MA). [γ-32P]ATP was purchased from ICN (Costa Mesa, CA). Texas Red-labeled zymosan and Lucifer Yellow were purchased from Molecular Probes ( Eugene, OR). PD098059 (2-amino-3-methoxyphenyl)-anaxaphenalen-4-one was synthesized as previously described (29). Bicarbonate-free medium RPMI 1640 was buffered to pH 7.3 with 25 mM Na-HEPES. The sodium-rich medium used for incubation of intact cells contained 140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES (pH 7.4). Both media were adjusted to 290 ± 5 mOsm with the major salt.

**Abs and recombinant proteins**

Polyclonal Abs recognizing MEK-1 were generated by immunizing rabbits with keyhole limpet hemocyanin coupled to the N-terminal sequence of MEK-1 (PKKPTPIQLNPPIPEY) and were gifts from Dr. Gilles L’Allemain (Centre de Biochimie, CNRS, Université de Nice, France). A mAb (IgG2a) to an N-terminal 13.6-kDa fragment of MEK-2 was obtained from Transduction Laboratories (Lexington, KY). Polyclonal Abs to ERK-1 and 2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Soluble and agaroose-coupled monoclonal anti-phosphotyrosine Abs (4G10) were obtained from Upstate Biochemicals, Inc. (Lake Placid, NY). Abs to the phosphorylated form of p38 MAPK were purchased from New England Biolabs, Inc. (Beverly, MA).

The cDNA construct encoding a GST fusion of the catalytically inactive form of ERK-1, in which Lys³⁸ → Thr²⁸ (K63M), was provided by Dr. R. L. Erikson (Department of Cellular and Developmental Biology, Harvard University, Boston, MA). GST-ERK-1 (K63M) fusion proteins, referred to hereafter simply as GST-ERK, were bacterially produced and purified on glutathione-agarose beads as described by Crews et al. (31).

**Neutrophil isolation**

Neutrophils were isolated from fresh heparinized blood from healthy human volunteers. The majority of the RBC were removed by dextran sedimentation, followed by Ficoll-Paque centrifugation. The remaining RBC were eliminated using hypotonic lysis. Neutrophils were counted using a model ZM Coulter counter (Coulter, Hialeah, FL), resuspended in HEPES-buffered RPMI 1640 at 10⁷ cells/ml, and maintained in this medium at room temperature until use. In experiments involving immunoprecipitation, the cells were pretreated with 2.5 mM di-isopropylfluorophosphate for 30 min at room temperature. Cell viability was estimated for all experiments using trypan blue exclusion and was always >95%.

**SDS-PAGE, immunoblotting, and ERK activity assay**

Immunoblotting was performed essentially as previously described (23), after separation of the proteins by SDS-PAGE (32) and transfer onto Immobilon (Millipore). Phosphorytorylated ERK was detected using a 1/5000 dilution of monoclonal anti-phosphotyrosine Abs, followed by a 1/5000 dilution of secondary, horseradish peroxidase-conjugated sheep anti-mouse Ab (Amersham, Arlington Heights, IL). The enhanced chemiluminescence (ECL) system from Amersham was used for detection. ERK activity was assayed after renaturation in gels polymerized in the presence of myelin basic protein as previously described (23, 24).

**Immunoprecipitation and MEK kinase assay**

Immunoprecipitation of phosphorytorylated MEK-1 and MEK-2 for in vitro kinase assays was performed as previously described (24). Briefly, MEK-1 or MEK-2 were immunoprecipitated under nondenaturing conditions and incubated with [γ-32P]ATP and GST-ERK as substrate. The kinase reaction was stopped with boiling Laemmli sample buffer, followed by SDS-PAGE. Substrate phosphorylation and the autophosphorylation of MEK-1 or MEK-2 were then quantified by phosphorimaging (see below). Experiments using nonimmune rabbit serum or isotype-matched (IgG2a) mAbs instead of the anti-MEK Abs and in the absence of primary Ab demonstrated that there was no phosphorylation of the GST-ERK substrate under these conditions (not shown).

**Oxidative burst**

NADPH oxidase activity was determined using the superoxide dismutase-inhibitable reduction of ferricytochrome c (33) as described previously (34). Treated cells (4 × 10⁶ cells/ml) were incubated in 1 ml of Na buffer with 75 μM cytochrome c at 37°C in the presence or the absence of superoxide dismutase. The cell suspensions were then placed in a stirred, thermostatted cuvette, and the absorbance was measured at 550 nm in a dual beam spectrophotometer (Hitachi, Tokyo, Japan). Stimulation of the cells with opsonized zymosan was accomplished by adding zymosan to the cells, and gently sedimenting the mixture in a microcentrifuge at room temperature, followed by resuspension of the cells in prewarmed assay buffer.

**Phagocytosis**

The phagocytic ability of polymorphonuclear leukocytes was assayed by incubating opsonized zymosan with cells in the presence of the impermeant fluid phase marker Lucifer Yellow. Cells (3 × 10⁶) were allowed to settle and adhere to fibrinogen-coated coverslips for 10 min at room temperature. To synchronize phagocytosis, the serum-opsonized zymosan (6 × 10⁶ particles) was added to the cells and allowed to bind for 10 min at 4°C. The temperature was then rapidly raised to 37°C, and incubation proceeded at this temperature for 3 min in the presence of Lucifer Yellow (2 mg/ml). The coverslips were immediately cooled using an ice-water bath. The number of phagosomes was counted using a fluorescence microscope (Nikon, Melville, NY). To ensure that the dye did not leave the phagosome, the cells were kept cool throughout the visualization period using a cooled

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microscope stage and precleaned slides. It is important to note that PD098059 did not alter the binding of opsonized zymosan to neutrophils.

Flow cytometric analysis of CD63 and CD67 surface expression

For experiments measuring FMLP-induced degranulation, to maximize the response, cells were pretreated with 5 μM cytochalasin B for 2 min before addition of the activating stimulus. After stimulation, cells were sedimented and resuspended in 200 μL of PBS followed by the addition of 800 μL of 1.6% paraformaldehyde in PBS. The cells were left on ice for 30 min followed by an additional 30 min at room temperature. After fixation, the cells were washed and resuspended in PBS containing 1% BSA. For both CD63 and CD67, the primary Ab was added at a dilution of 1/400 for 2 h at room temperature in the presence of 1% BSA. The cells were again washed with 1% BSA in PBS, and the secondary, FITC-labeled, goat anti-mouse Ab was added at a 1:400 dilution. After 1 h at room temperature, the cells were washed and resuspended in 0.5 mL of PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

Actin polymerization

Actin polymerization was determined using the fluorescent marker 7-nitrobenz-2-oxa-1,3-diazole (NBD)phallacidin as described by Howard and Meyer (35). Briefly, after stimulation, the cells were fixed and permeabilized in PBS containing 3.7% formaldehyde and 1 mg/mL t-cam-bromelain and washed with 1% BSA in solution for 5 min at 37°C. NBD-phallacidin was added to the cells at a final concentration of 1.65 x 10^{-7} M for 10 min at 37°C. The intensity of staining was analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

Chemotaxis

Chemotaxis was measured using a micro-Boyden chamber (Neuroprobe, Cabin John, MD). The chamber consists of two wells separated from each other by filter paper. The chemotactrant (10^{-7} M FMLP in HEPES-buffered RPMI with 1% BSA at pH 7.4) was placed in the bottom well, and a 0.45-μm pore size trap filter was placed above, followed by a 3-μm pore size chemotaxis filter. The top chamber was secured in place, and the cells were added in HEPES-buffered RPMI with 1% BSA (3 x 10^6 cells/well). The chamber was incubated at 37°C for 2 h; the trap filter was removed, fixed, and stained with hematoxylin; and the number of cells present was counted.

Lipid extraction and measurement of arachidonate production

Extracellular release of arachidonic acid (AA) from polymorphonuclear leukocytes was used as an index of phospholipase A2 activity. Cells were incubated in NaCl-rich medium containing 0.1% BSA to trap extracellular arachidonate. After agonist exposure, ice-cold KRPD was added to the cells followed by rapid centrifugation. After addition of the internal standard (deuterated arachidonic acid), supernatants were extracted with 6 mL of chloroform/methanol (2/1, v/v), with 0.01% butyated hydroxytoluene as an antioxidant. The lower phase was collected, and the upper phase was re-extracted twice with chloroform/methanol/0.9% NaCl (86/14/1, v/v/v). The pooled lower phase was dried under N2, reconstituted with hexane/methyl-tart-butyl ether (98/2, v/v), and applied to a Sep-Pak silic acid column (Waters, Toronto, Ontario, Canada). After elution of individual lipid classes with solvent mixtures of increasing polarity, the fatty acid fraction was dried under N2 and derivatized with N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide. The t-butyldimethylsilyl ether of AA was then separated from other fatty acids on a 0.2-mm x 25-m DB-23 gas chromatography column and quantified by selective ion monitoring mass spectrometry, by monitoring the intensities of the M+7 ions at m/z 361 (AA) and 369 (deuterated arachidonic acid). The assay for AA was linear between 0.1 and 400 pM (r^2 = 0.996; p < 0.001).

Apoptosis

Apoptosis was measured using propidium iodide staining according to the method of Nicoletti et al. (36). After preparation, the cells were suspended in HEPES-buffered RPMI containing 10% FCS at a concentration of 2.5 x 10^6 cells/mL and incubated in polypropylene tubes for approximately 24 h. Subsequently, the cells were sedimented and resuspended in 0.5 mL of a hypotonic solution (3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, and 0.1% Triton X-100) containing 50 μg/mL of propidium iodide. After a 30-min incubation at 4°C in the dark, nuclear fluorescence was quantified by flow cytometry (FACScan, Becton Dickinson).

Other methods

Radioactivity incorporated into MEK or GST-ERK was quantified by phosphorimaging (Molecular Dynamics, Sunnyvale, CA), using ImageQuant software. For reproduction, the images were saved as 16-bit TIF files, cropped using Adobe Photoshop, and labeled using Adobe Illustrator (Adobe Systems Inc., Mountain View, CA). For illustrations, immunoblots were scanned by a Hewlett-Packard Jet Scan II cx using Desk Scan II v2.1 software (Hewlett-Packard, Palo Alto, CA). For quantitation, immunoblots were scanned using a model DNA 35 high resolution flatbed scanner and analyzed using the PDI one-dimensional gel analysis software (Protein Databases Inc., Huntington Station, NY). Results are presented as typical radiograms or fluorescence traces or as the mean ± SE of the indicated number of replicates. Statistical analysis was performed using Student’s t-test for paired or unpaired data or analysis of variance as indicated.

Results and Discussion

**PD098059 inhibits activation of MEK-1 and -2**

We have recently demonstrated that human neutrophils express MEK-1 and MEK-2, and that these enzymes are activated in response to diverse soluble agonists such as FMLP and TPA (24). To determine whether activation of MEKs could be inhibited by PD098059, human peripheral blood neutrophils were incubated with the compound before exposure to FMLP. Figure 1 illustrates that treatment with ≥50 μM PD098059 inhibited FMLP-induced activation of both MEK-1 and MEK-2 almost completely (>90%), as determined by an in vitro immune complex kinase assay using recombinant, kinase-deficient ERK-1 as the substrate. These results confirm that this compound is both membrane permeant and effective in neutrophils. Figure 1B illustrates the dose-dependent inhibition of MEK1 by PD098059: the IC_{50} for MEK1 was approximately 3.5 μM, comparable to values (~4 μM) obtained in other cell types (29, 30). It should be noted that PD098059 inhibited both the autophosphorylating activity (solid arrowhead in Fig. 1A) as well as the ability of MEK-1 and -2 to phosphorylate recombinant kinase-deficient ERK-1 (open arrowhead in Fig. 1A). To ensure the specificity of PD098059, the effects of the compound on another member of the MAPKsuperfamily, p38 MAPK, was assayed. Figure 1C illustrates that 50 μM PD098059 had minimal effects (~30%) on FMLP-induced phosphorylation of p38 MAPK, confirming the specificity of the inhibitor.

As activation of neutrophils is known to be induced by diverse agents acting via distinct signaling pathways, we next sought to determine whether these agonists also induced MEK activation. Figure 1D illustrates that exposure of neutrophils to the phorbol ester TPA (a direct activator of protein kinase C), opsonized zymosan (a particulate stimulus acting via Fc and CR3 receptors), and GM-CSF (acting via a tyrosine kinase-linked growth factor receptor) induced MEK1 activation, and this was substantially (from 60–90%) inhibited by PD098059.

**PD098059 inhibits tyrosine phosphorylation and activation of ERK-1 and -2**

MEKs are dual specificity kinases that phosphorylate substrates on both Tyr and Thr residues (17). The purported physiologic substrates of MEK-1 and -2 are ERKs, members of the MAPK family (31). Human neutrophils have been shown to express both ERK-1 and -2, and these enzymes are activated in cells exposed to chemotactractants (10). To determine the effects of MEK inhibition by PD098059 on the activation of ERK-1 and -2 in intact cells, neutrophils were incubated with the compound before chemotactractant exposure, as described above. The cells were then lysed, and the phosphorylation and activity of ERK-1 and -2 were evaluated. PD098059 prevented the tyrosine phosphorylation of both ERK-1 and -2 induced by FMLP (Fig. 2A). Moreover, at concentrations of ≥50 μM, the compound almost completely (>90%) abrogated the
enhanced activity of ERK-1 and -2 as assayed by an in-gel renaturation assay using myelin basic protein as the substrate (Fig. 2, B and C). A similar pattern of inhibition of ERK-2 was noted using immunoprecipitation followed by an in vitro kinase assay using myelin basic protein as the substrate (data not shown). The IC$_{50}$ for FMLP-induced ERK-1 and ERK-2 activation was about 6 μM, comparable to that for MEK inhibition (cf., Fig. 1B). Together, these observations are consistent with the idea that MEK-1 and/or MEK-2 are the predominant upstream activators of ERK-1 and -2 in response to diverse agonists.

**PD098059 inhibits activation of the oxidative burst**

As discussed above, the MEK-ERK pathway has been tentatively implicated in the activation of certain microbicidal functions of neutrophils, such as the oxidative burst. Figure 3, A and B, shows that treatment of neutrophils with 50 μM PD098059 substantially (~70%) inhibited the FMLP-induced oxidative burst. The inhibition was statistically significant, using Student’s paired t test ($p < 0.05$). Perusal of traces such as that in Figure 3A revealed that the MEK inhibitor altered mainly the rate of oxidant production, with little effect on the duration of the burst. As the magnitude of the...
significant inhibitory effects on the chemoattractant-induced oxidative burst.

We next studied the effects of the MEK inhibitor on the oxidative burst induced by alternate agonists. Figure 3C illustrates that PD098059 significantly inhibited the respiratory burst induced by the phagocytic stimulus, opsonized zymosan. These findings imply that although soluble and particulate agonists use different signal transduction pathways (37), they may share some of the downstream events. In contrast to the formyl peptide and opsonized zymosan, the respiratory burst in response to TPA was minimally inhibited by PD098059 (Fig. 3D). These results underscore two important points. First, the MEK inhibitor does not inhibit the NADPH oxidase directly. Second, although the phorbol ester activates both MEK and ERK, activation of the oxidative burst in response to this potent agonist may proceed by alternate (perhaps parallel) pathways.

PD098059 partially inhibits phagocytosis of opsonized zymosan

Another key component of the microbicidal function of neutrophils is their ability to ingest foreign organisms by phagocytosis. To determine whether the MEK-MAPK pathway is involved in regulation of this important function, the effects of PD098059 on the phagocytosis of opsonized zymosan were analyzed. Figure 4 illustrates the experimental protocol as well as the results of such determinations. Phagocytosis was quantified by a combination of phase-contrast and fluorescence microscopy. Successful phagosome formation was scored by assessing the number of Texas Red-labeled yeast particles that were colocalized with the trapped fluid phase marker Lucifer Yellow. This procedure allowed us to clearly discern extracellular zymosan (arrows in Fig. 4, B–D) from those trapped within the cells (arrowheads). As illustrated in Figure 4A, compound PD098059 induced a partial (~25%), yet significant, inhibition of phagocytosis. Importantly, PD098059 did not diminish binding of the opsonized zymosan to neutrophils.

PD098059 does not inhibit exocytosis of primary or secondary granules

Another important facet of the bactericidal function of neutrophils is the regulated exocytosis of the contents of the various secretory granules (3). To ascertain the importance of the MEK-MAPK pathway in the secretion of granular contents, we used immunofluorescence and flow cytometry to quantify the surface expression of granule membrane markers (38). CD63 was used as a marker of primary granule secretion, while CD67 (CD66a) was used as a marker for secondary granules. The summarized data from three such experiments of each kind are presented in Figure 5, A and B. Treatment of neutrophils with either FMLP or opsonized zymosan resulted in increased plasma membrane expression of markers for both primary (CD63) and secondary (CD67) granules, in good agreement with earlier findings (39). This increased surface expression of granule markers detected by immunofluorescence has been shown to correlate closely with conventional assays of granular content release (38, 39). The effect of PD098059 was tested next. At concentrations that virtually eliminated the activity of MEK, the inhibitor (50 μM) had no significant effect on the exocytosis of primary (Fig. 5A) or secondary (Fig. 5B) granules in response to either soluble (FMLP) or particulate (zymosan) stimuli.

PD098059 does not inhibit actin polymerization or chemotaxis

Neutrophils must be able to emigrate from the vascular space, across the endothelium, and through interstitial tissues to a site of

FMLP-induced oxidative burst is relatively small, experiments such as that illustrated in Figure 3A were performed in cells pre-treated with cytochalasin B, an actin-disrupting agent known to enhance the response to the chemoattractant (8, 34). To ensure that the inhibitory effects of PD098059 were exerted on FMLP-activated pathways (and not simply blocking the enhancement due to cytochalasin B), the experiments were repeated in the absence of cytochalasin B. Figure 3B illustrates that although the magnitude was lower in the absence of cytochalasin, PD098059 still exerted

FIGURE 2. Activation of ERK-1 and ERK-2 in the presence of PD098059. Samples were pretreated with the indicated concentration of PD098059 and stimulated with FMLP as described in Figure 1. A, Tyrosine phosphorylation of ERK-1 and -2. Anti-phosphotyrosine (anti-PY) immunoprecipitates were subjected to SDS-PAGE, transferred to Immobilon membranes, and blotted with anti-ERK-1- or anti-ERK-2-specific Abs. The results shown are representative of three experiments. The presence of two bands after anti-PY immunoprecipitation followed by immunoblotting with anti-ERK 2 Abs has been reported by both ourselves and others previously. While the upper band could conceivably represent a contribution from ERK-1, it more likely represents a hyperphosphorylated form of ERK-2. B, Effects of FMLP and PD098059 on the kinase activity of ERK-1 (open arrow) and ERK-2 (solid arrow). Cells were pretreated with the indicated dose of PD098059, and ERK activity was determined by an in-gel renaturation assay using myelin basic protein as a substrate. C, Quantitation of ERK-1 and ERK-2 inhibition by PD098059. Experiments such as that shown in B were quantified by phosphorimaging, and after subtracting the background (unstimulated) activity, the maximal activity, recorded in FMLP-stimulated samples without inhibitor, was normalized to 100%. Data points represent the mean ± SE of three experiments.
infection. This requires coordinated cell movement along a gradient of attractant molecules that is termed chemotaxis (40). The coordinated assembly, disassembly, and redistribution of the actin cytoskeleton is crucial for this motile response (reviewed in Ref. 40). To determine the involvement of MEK and MAPK in these responses, we studied the effects of PD098059 on actin assembly, measured by binding of NBD-phallacidin staining and flow cytometry. In Figure 6, representative flow cytometric histograms illustrate the increase in F-actin induced by exposure of neutrophils to either FMLP or opsonized zymosan. Figure 6 summarizes data from three similar experiments in which the effect of PD098059 was evaluated. The assembly of F-actin induced by either the soluble or particulate agonists was essentially unaffected by PD098059.

The effect of PD098059 on chemotaxis was also measured, using micro-Boyden chambers. As detailed in Figure 7, the strong chemoattractant effect exerted by FMLP was not significantly affected by PD098059 under conditions shown previously to inhibit activation of both MEK-1 and -2 as well as ERK.

PD098059 does not prevent stimulation of phospholipase A₂

Activation of phospholipase A₂ is an early and purportedly important response of stimulated neutrophils (41). Activation of this enzyme yields arachidonate, which has been implicated in cytoskeletal alterations (42) and in triggering the oxidative burst (43). It has been demonstrated that ERKs are able to phosphorylate and activate phospholipase A₂ (26). If ERKs are the prevailing upstream activators of phospholipase A₂ in neutrophils, then inhibition of MEK (and therefore ERK) would be predicted to diminish or abrogate arachidonate production. This possibility was analyzed by a combination of gas chromatography and mass spectrometry. In accordance with earlier findings, both FMLP and the phorbol ester TPA triggered the release of arachidonate from human neutrophils (Fig. 8). Treatment of unstimulated cells with PD098059 resulted in a significant elevation in arachidonate release. More importantly, treatment with PD098059 did not alter the net amount of arachidonate released by either FMLP or TPA (Fig. 8). These
observations suggest that ERK-1 and -2 are not the main activators of phospholipase A2 in stimulated neutrophils.

**PD098059 partially prevents the effects of GM-CSF on apoptosis**

Apoptosis is a process of programmed cell death that results in degradation and fragmentation of chromosomal DNA. Apoptosis is also accompanied by alterations in the cell membrane that allow recognition and clearance of effete inflammatory cells by macrophages, a process of major importance in the resolution of inflammation (44). Peripheral blood neutrophils, in their unactivated state, are relatively short lived ($t_{1/2} = 6–8$ h) and undergo spontaneous apoptosis when maintained in culture (44). However, exposure to cytokines such as GM-CSF has been shown to delay apoptosis (45), thereby prolonging the half-life of these cells, potentially contributing to host defense or, under other circumstances, tissue injury (46). It is noteworthy that GM-CSF exposure has also been reported to activate ERKs (47) and that recent evidence points to a role of these kinases in preventing or delaying apoptosis (48, 49). Figure 9A confirms the former findings and additionally illustrates that PD098059 effectively inhibits the tyrosine phosphorylation of both ERK-1 and -2 induced by GM-CSF. To determine whether ERK activation was involved in the delay of apoptosis, nucleic acid fragmentation was estimated by staining with propidium iodide and flow cytometry. The cells were incubated with PD098059 before and during exposure to GM-CSF, and apoptosis was measured 24 h later. As detailed in Figure 9B, the cytokine substantially reduced the fraction of apoptotic cells.
More importantly, PD098059 increased the number of apoptotic cells observed after a 24-h incubation in the presence of GM-CSF. The MEK inhibitor did not completely reverse the effect of GM-CSF, but the increase noted was statistically significant ($p < 0.05$).

### Concluding remarks

Reports from many groups have documented a prominent activation of the MEK/MAPK pathway after exposure of neutrophils to diverse inflammatory mediators (8, 10, 23, 24, 50, 51). Although the activation of these kinases was found to precede or parallel several physiologic responses, no definitive proof of a causal relationship between these events was provided. In principle, the role of specific kinases in defined responses can be studied by transfection or microinjection of constitutively activated or dominant negative (interfering) mutants. These approaches, however, are not applicable to neutrophils. These are terminally differentiated cells with a remarkably short biologic half-life, precluding transfection. Myeloid cell lines are notoriously difficult to transfect and have phenotypes that do not appropriately reflect the function of mature neutrophils. Microinjection is similarly intractable; neutrophils are small, pliable, and react to contact with glass.

An alternative strategy is to use specific, cell-permeant, pharmacologic inhibitors with adequate specificity toward a defined enzyme. The present study used this approach, taking advantage of the well-documented selectivity of PD098059, an antagonist of MEK activation. The resulting findings suggest that the MEK-MAPK pathway provides substantial input into some important effector responses of neutrophils, including the oxidative burst and phagocytosis. These conclusions are in agreement with a recent report by Avdi et al. (52), which appeared while this work was in progress, that treatment of neutrophils with PD098059 partially inhibited the FMLP-induced respiratory burst. It should be noted that a correlation between ERK activation and activation of the NADPH oxidase has not been observed by all investigators. For example, Yu et al. (53) recently reported that increased intracellular cAMP resulted in inhibition of the FMLP-stimulated oxidative burst, whereas MAPK activity remained under these conditions. However, it is possible that only partial activity of MAPK (perhaps of a minor fraction or isoform) suffices for activation of the oxidative burst. Alternatively, multiple pathways, each capable of independent input, may contribute to initiation of the oxidative burst. Indeed, our observations that PD098059 did not completely inhibit superoxide generation or phagocytosis, while virtually obliterating MEK and ERK activity, suggests that multiple, possibly redundant, signals can initiate these responses. That the respiratory burst can be triggered by a variety of seemingly unrelated second messengers, including calcium, agonists of protein kinase C, arachidonate and other lipid metabolites, and agents that favor phosphotyrosine accumulation (54–57) provides additional support for this idea. Recent studies have indicated that another MAPK family member, p38 MAPK, may provide input into the

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Effect of PD098059 on actin polymerization. Neutrophils were preincubated with or without PD098059, followed by stimulation with either FMLP or opsonized zymosan. Actin polymerization was determined using the fluorescent marker NBD-phallacidin. Data are the mean ± SE of three experiments. RFI, relative fluorescence intensity, normalized to the value of untreated cells.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Effect of PD098059 (PD) on chemotaxis. Cells were preincubated with or without PD098059 as described in Figure 1. FMLP-induced chemotaxis of neutrophils was then assessed in a micro-Boyden chamber. The number of neutrophils that travelled through a 3-μm pore size filter and were then trapped in a 0.45-μm pore size filter was determined after hematoxylin staining. Data are the mean ± SE of seven separate experiments.

More importantly, PD098059 increased the number of apoptotic cells observed after a 24-h incubation in the presence of GM-CSF. The MEK inhibitor did not completely reverse the effect of GM-CSF, but the increase noted was statistically significant ($p < 0.05$).

**Concluding remarks**

Reports from many groups have documented a prominent activation of the MEK/MAPK pathway after exposure of neutrophils to diverse inflammatory mediators (8, 10, 23, 24, 50, 51). Although the activation of these kinases was found to precede or parallel several physiologic responses, no definitive proof of a causal relationship between these events was provided. In principle, the role of specific kinases in defined responses can be studied by transfection or microinjection of constitutively activated or dominant negative (interfering) mutants. These approaches, however, are not applicable to neutrophils. These are terminally differentiated cells
signaling pathways leading to activation of the oxidative burst (58, 59), adherence, and chemotaxis (58). As there are multiple phosphorylation sites on p47phox (2), it is conceivable that phosphorylation of this cytosolic component by both ERKs and p38 MAPK is required for maximal activation of the oxidase.

The use of a pharmacologic inhibitor in the current study engenders certain limitations pertaining primarily to issues of specificity. For example, PD908059 had a small, but consistent, inhibitory effect on p38 MAPK phosphorylation (Fig. 1C). Nonetheless, it is unlikely that this small effect of the MEK inhibitor on p38 MAPK could have accounted for the inhibition of the oxidative burst or phagocytosis, since complete inhibition of p38 MAPK by a more potent and specific inhibitor (SB203580) had no effect on these microbicidal functions (60).

Our findings that PD908059 attenuated the GM-CSF-induced delay in apoptosis has important implications for the regulation of the inflammatory process. In an inflammatory response initiated by bacterial infection, prolongation of leukocyte survival might be expected to facilitate the killing of invading microbes by these phagocytic cells. On the other hand, in circumstances of inflammatory-mediated tissue damage such as the systemic inflammatory response syndrome (53), the persistence of tissue neutrophils might be undesirable. Clearly, the fate of inflammatory cells must be carefully regulated (44). Our results suggest that the delay of apoptosis induced by cytokines such as GM-CSF is mediated in part by activation of the MEK-MAPK pathway. This idea is in good agreement with recent observations in cells of neural origin, in which apoptosis induced by withdrawal of nerve growth factor was found to be mediated by activation of Jun kinase and p38 MAPK in concert with inhibition of p42ERK and p44ERK (48). It follows from the above considerations that by accelerating the apoptosis of tissue neutrophils, judicious use of MEK-ERK inhibitors might afford protection against inflammatory tissue injury (44). Conversely, the use of these inhibitors is not indicated when the microbicidal function of the leukocytes is more important.

In summary, the inhibitor PD908059 largely prevented chemotactic-induced activation of MEK-1 and -2 and of ERK-1 and -2, and substantially inhibited the oxidative burst and phagocytosis. Other neutrophil functional responses, including exocytosis, actin polymerization, and motility, as well as activation of phospholipase A2 were relatively unaffected by the compound. Importantly, PD908059 prevented the delay of apoptosis promoted by the growth factor GM-CSF. We conclude that activation of MEK and ERK provides substantial input into the signaling pathways regulating the oxidative burst, phagocytosis, and apoptosis. Other pathways appear to be largely responsible for secretion, actin assembly, chemotaxis, and release of arachidonate by neutrophils. These results demonstrate that distinct intracellular signaling pathways lead to activation of the various responses and highlight the ability of neutrophils to differentially regulate these important microbicidal functions.

References
