Neutrophil superoxide production can be potentiated by prior exposure to “priming” agents such as granulocyte/macrophage colony-stimulating factor (GM-CSF). Because the mechanism underlying GM-CSF-dependent priming is not understood, we investigated the effects of GM-CSF on the phosphorylation of the cytosolic NADPH oxidase components p47\textsuperscript{phox} and p67\textsuperscript{phox}. Preincubation of neutrophils with GM-CSF alone increased the phosphorylation of p47\textsuperscript{phox} but not that of p67\textsuperscript{phox}. Addition of formyl-methionyl-leucyl-phenylalanine (fMLP) to GM-CSF-pretreated neutrophils resulted in more intense phosphorylation of p47\textsuperscript{phox} than with GM-CSF alone and fMLP alone. GM-CSF-induced p47\textsuperscript{phox} phosphorylation was time- and concentration-dependent and ran parallel to the priming effect of GM-CSF on superoxide production. Two-dimensional tryptic peptide mapping of p47\textsuperscript{phox} showed that GM-CSF induced phosphorylation of one major peptide. fMLP alone induced phosphorylation of several peptides, an effect enhanced by GM-CSF pretreatment. In contrast to fMLP and phorbol 12-myristate 13-acetate, GM-CSF-induced phosphorylation of p47\textsuperscript{phox} was not inhibited by the protein kinase C inhibitor GF109203X. The protein tyrosine kinase inhibitor genistein and the phosphatidylinositol 3-kinase inhibitor wortmannin inhibited phosphorylation of p47\textsuperscript{phox} induced by GM-CSF and by fMLP but not that induced by phorbol 12-myristate 13-acetate. GM-CSF alone did not induce p47\textsuperscript{phox} or p67\textsuperscript{phox} translocation to the membrane, but neutrophils treated consecutively with GM-CSF and fMLP showed an increase (compared with fMLP alone) in membrane translocation of p47\textsuperscript{phox} and p67\textsuperscript{phox}. Taken together, these results show that the priming action of GM-CSF on the neutrophil respiratory burst involves partial phosphorylation of p47\textsuperscript{phox} on specific serines and suggest the involvement of a priming pathway regulated by protein-tyrosine kinase and phosphatidylinositol 3-kinase.

EXPERIMENTAL PROCEDURES

Reagents—fMLP, proteases and phosphatases inhibitors were from Sigma. \textsuperscript{32}POrthophosphoric acid was from NEN Life Science Products. Kinases inhibitors were from Calbiochem. Injection-grade water and 0.9% NaCl were endotoxin-free (<0.4 pg/ml) in the limulus test

* This work was supported by a grant from Recherches et Partages. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Contributed equally to this work.

§ To whom correspondence should be addressed. Tel.: 33 1 40 25 85 21; Fax: 33 1 44 85 62 07; E-mail: benna@bichat.inserm.fr.

Human polymorphonuclear neutrophils play a key role in host defenses against invading microorganisms. In response to a variety of stimuli, neutrophils release large quantities of superoxide anion (O_2\textsuperscript{-}) in a phenomenon known as the respiratory burst. O_2\textsuperscript{-} is the precursor of potent oxidants, which are essential for bacterial killing and also potentiate inflammatory reactions (1). Neutrophil production of O_2\textsuperscript{-} is dependent on the respiratory burst oxidase, or NADPH oxidase, a multicomponent enzyme system that catalyzes NADPH-dependent reduction of oxygen to O_2\textsuperscript{-} (2, 3). NADPH oxidase is activated and regulated by various neutrophil stimuli at infectious or inflammatory sites. Proinflammatory cytokines such as GM-CSF,\textsuperscript{1} tumor necrosis factor, and interleukin-8 modulate NADPH oxidase activity through a priming phenomenon (4–6). These cytokines induce a very weak oxidative response by neutrophils but strongly enhance neutrophil release of reactive oxygen species on exposure to a secondary applied stimulus such as bacterial N-formyl peptides (7, 8). The mechanisms underlying the priming process are poorly understood, although some studies have suggested that priming with various agonists is regulated at the receptor and post-receptor levels (7, 9, 10).

DeLeo et al. (11) recently reported that priming of the respiratory burst by lipopolysaccharide resulted in limited phosphorylation of p47\textsuperscript{phox} as well as redistribution of oxidase components. However, phosphorylation of NADPH-oxidase components during GM-CSF priming process has not been studied.

In resting cells, NADPH oxidase is inactive, and its components are distributed between the cytosol and membranes. When cells are activated, the cytosolic components (p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox}, and Rac2) migrate to the membranes, where they associate with the membrane-bound component (flavocytochrome b 558) to assemble the catalytically active oxidase (12–16). Upon oxidase activation, p47\textsuperscript{phox} and p67\textsuperscript{phox} become phosphorylated (17–19). p47\textsuperscript{phox} phosphorylation on several serines plays a pivotal role in oxidase activation in intact cells (20–24). Different kinases have been shown to phosphorylate p47\textsuperscript{phox} in vitro, but the regulatory pathways involved in different conditions of stimulation in vivo are unknown.

To further define the mechanisms involved in proinflammatory cytokine-induced priming of the neutrophil respiratory burst triggered by fMLP, we analyzed the effects of GM-CSF alone and combined with fMLP on the phosphorylation of p47\textsuperscript{phox} and p67\textsuperscript{phox}.
(Charles River, Charlestone, SC). Endotoxin-free buffers and salt solutions were from Life Technologies, Inc. Recombinant human GM-CSF was a generous gift from Dr. J. J. Garaud (Scherling-Plough Research Institute, Kenilworth, New Jersey). Rabbit polyclonal antibody against p47phox was a generous gift from Dr. B. M. Babior (Scripps Research Institute, CA).

**Neutrophil Preparation**—Human neutrophils were obtained in lipid-polyacrylamide-sensitive conditions by means of dextran sedimentation and Ficoll centrifugation as described previously (19), except that diisopropylfluorophosphate treatment was omitted, as we found that diisopropylfluorophosphate alone from some lots can increase p47phox phosphorylation.

**Measurement of O2 Production**—Neutrophils were treated with GM-CSF at 37 °C at various concentrations and incubation times in Hank’s buffered saline solution plus 0.25% endotoxin-free bovine serum albumin. Thereafter, O2 production was measured in response to fMLP (10-7 M) in terms of superoxide dismutase-inhibitable ferricytochrome c reduction as described previously (16).

**P2 Labeling of Neutrophils, Stimulation, and Fractionation**—Cells were incubated in phosphate-free buffer containing 0.5 mCi of [32P]orthophosphoric acid/108 cells/ml for 60 min at 30 °C, as previously reported (19). Neutrophils were then treated with GM-CSF, as described above, and stimulated or not with 10-7 M fMLP for a further 2 min. The reaction was stopped by adding ice-cold buffer and centrifugation at 400 × g for 6 min at 4 °C. The cells were lysed by resuspending them in lysis buffer, as previously described (19). The suspension was sonicated on ice for 3 × 15 s. The lystate was centrifuged at 100,000 × g for 30 min at 4 °C in a TL100 Ultracentrifuge (Beckman).

**Immunoprecipitation of p47phox and p67phox**—The cleared supernatant was incubated overnight with anti-p47phox (1/200) or anti-p67phox (1/150) antibodies; protein was then immunoprecipitated using Gamma-bind G-Sepharose beads (Amersham Pharmacia Biotech) and washed as described previously (19, 23).

**Preparation of Neutrophil Membranes**—Neutrophils were preincubated with GM-CSF (500 pm) for 20 min in Hank’s buffered saline solution containing 0.25% bovine serum albumin and 1 mM NaN3, then stimulated with fMLP (10-7 M). Membranes were then prepared as described by Abo et al. (15); briefly, 1 × 108 neutrophils/ml were sonicated for 3 × 5 s on ice in 1 ml of relaxation buffer (10 mM Pipes, pH 7.3, 3 mM MgCl2, 100 mM KCl, 5 mM NaCl) supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 10 µg/ml leupeptin, and 10 µg/ml pepstatin. Cytosolic and membrane fractions were separated by centrifugation at 200,000 × g for 15–34% (w/v) sucrose gradient for 30 min at 4 °C.

**Electrophoresis and Blotting**—The samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 10% polyacrylamide gels, using standard techniques (25, 26). The separated proteins were transferred to nitrocellulose and detected as described elsewhere (16, 19).

**Two-dimensional Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis**—Tryptic digestion of p47phox on nitrocellulose, thin-layer electrophoresis, and thin-layer chromatography were performed as described previously (22, 24). The nitrocellulose area containing 32P-labeled p47phox was incubated for 30 min at 37 °C with polyvinylpyrrolidone, washed, then incubated overnight with trypsin (50 µg/ml) in carbonate buffer. Released peptides were washed three times in a Speed-Vac, redissolved in electrophoresis buffer (17 volumes of water:3 volumes of 88% formic acid), and applied to one corner of a cellulose thin-layer plate (Merck). After electrophoresis (1000 V for 20 min), chromatography was performed as described previously (23, 24). The plates were autoradiographed for 1 week at -75 °C. Phosphoamino acid analysis was performed as described previously (19).

**Statistical Analysis**—All results are expressed as means ± S.E. Significant differences were detected using the Student’s t test.

---

**RESULTS**

**GM-CSF Induces Phosphorylation of p47phox but Not of p67phox**—Fig. 1 (lower panel) shows that GM-CSF alone did not induce superoxide production; in contrast, it potentiated the respiratory burst induced by 10-7 M fMLP, in accordance with the literature (7–10). To determine the effect of GM-CSF on p47phox and p67phox phosphorylation, 32P-labeled neutrophils were treated with GM-CSF (500 pm for 20 min) and then stimulated with 10-7 M fMLP. p47phox and p67phox were immunoprecipitated with specific antibodies and then analyzed by SDS-PAGE, Western blot, and autoradiography. As shown in Fig. 1 (upper panel), p47phox phosphorylation in GM-CSF-treated neutrophils was clearly higher than in untreated cells (202.7 ± 8.5% of control untreated cells (n = 4, p < 0.01) as determined by densitometry analysis); fMLP (10-7 M) also stimulated the phosphorylation of p47phox (210.5 ± 15% of control (n = 4, p < 0.01). Pretreatment of neutrophils with GM-CSF and subsequent stimulation with fMLP resulted in stronger phosphorylation than that induced by each agent alone (410.5 ± 17.7% of control (n = 4, p < 0.01)). Western blot analysis (middle panel) showed that the same amount of p47phox was immunoprecipitated from each sample. p47phox phosphorylation was not detected when irrelevant control antibodies were used (data not shown). Because p67phox is also phosphorylated during NADPH oxidase activation (18, 19), we analyzed the effect of GM-CSF on its state of phosphorylation. p67phox phosphorylation was not increased by GM-CSF alone (500 pm) relative to resting cells. In addition, GM-CSF did not increase the p67phox phosphorylation induced by fMLP (10-7 M) (data not shown).

**Comparison between GM-CSF-induced p47phox Phosphorylation and the GM-CSF Priming Effect on Superoxide Production**—To attempt to establish a relationship between p47phox phosphorylation and the priming effect of GM-CSF on superoxide production, we performed parallel kinetics and concentration-effect studies on these two processes. As shown in Fig. 2, p47phox phosphorylation increased with the GM-CSF incubation time. The time course of p47phox phosphorylation ran closely parallel to the kinetics of GM-CSF-primed superoxide production in response to fMLP (10-7 M). In addition, a concen-
GM-CSF Priming and p47phox Phosphorylation

The pathway controlling p47phox phosphorylation in response to GM-CSF is totally different from that underlying the action of PMA and might belong to the multiple pathways used by fMLP.

Characterization of Phosphorylated Amino Acids on p47phox—We characterized the phosphorylated amino acids on p47phox isolated from GM-CSF-treated neutrophils. Fig. 6 shows that p47phox was phosphorylated only on serines. In addition, no phosphorylated tyrosines were detected by Western blotting using an anti-phosphotyrosine antibody (data not shown). This result suggests that a genistein-sensitive protein-tyrosine kinase is an upstream regulator of a serine/threonine kinase that phosphorylates p47phox.
FIG. 5. Effect of protein kinase inhibitors on p47phox phosphorylation. 32P-Labeled neutrophils were incubated without (Control) or with 5 μM GF109203X (Gen.), 100 μM genistein (Gen.), or 250 nM wortmannin (Wort.) for 15 min, then with 500 pM GM-CSF for 20 min (A) or with fMLP (10−6 M) or PMA (0.5 mg/ml) for 3 and 8 min, respectively (B). p47phox was then immunoprecipitated from the lysate as described under “Experimental Procedures.” Proteins were analyzed by SDS-PAGE, transferred and analyzed by autoradiography, then by immunodetection using an anti-p47phox antibody. Data are representative of three different experiments.

FIG. 6. GM-CSF induces the phosphorylation of p47phox on serine residues. 32P-Labeled p47phox from GM-CSF-treated neutrophils was hydrolyzed in HCl. The resulting phosphoamino acids were analyzed by one-dimensional high voltage electrophoresis and autoradiography. The position of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr), determined by ninhydrin staining of standards, is indicated. Data are representative of three experiments.

GM-CSF Does Not Induce but Enhances fMLP-induced Translocation of p47phox and p67phox—NADPH oxidase assembly at the membrane site is dependent on complete phosphorylation of p47phox (17, 27). We observed that the partial phosphorylation of p47phox induced by GM-CSF was insufficient to induce p47phox or p67phox translocation (Fig. 7). However, neutrophil priming by GM-CSF followed by stimulation with 10−7 M fMLP resulted in stronger translocation than that observed with fMLP alone (163.8 ± 13.8% and 177.5 ± 10.6% control (fMLP alone) (n = 4, p < 0.01) for p47phox and p67phox, respectively, as determined by densitometry analysis). This suggested that prephosphorylation of p47phox induced by GM-CSF may accelerate the completion of p47phox phosphorylation on other sites and the movement of the cytosolic subunits to membranes.

DISCUSSION

GM-CSF alone induced partial phosphorylation of the cytosolic oxidase component p47phox, suggesting a role of this proc-ness in the priming effect of GM-CSF on the respiratory burst. The kinetics and dose-effect studies of GM-CSF-induced p47phox phosphorylation was parallel to GM-CSF-induced priming of superoxide production by neutrophils in response to fMLP 10−7 M. This phosphorylation was distinct from that induced by PMA, which phosphorylates other peptides and the sensitivity of which to protein kinase inhibitors (GF109203X, genistein, and wortmannin) was totally different. In addition, the partial GM-CSF-induced p47phox phosphorylation differed from fMLP-induced phosphorylation, as it was insensitive to GF109203X, although it was also inhibited by genistein and wortmannin. These results suggest that the GM-CSF priming pathway might share one of the multiple pathways used by fMLP signaling. Furthermore, GM-CSF increased both the degree of phosphorylation of p47phox peptides and the translocation of p47phox and p67phox when fMLP was subsequently added at a low concentration. These results suggest that GM-CSF triggers phosphorylation of only certain serines on p47phox that represent the first cytosolic step in the phosphorylation cascade, as GM-CSF alone did not induce p47phox or p67phox membrane translocation. Stepwise phosphorylation of p47phox has previously been suggested on the basis of two-dimensional gel analysis in cytochrome b-deficient neutrophils (17) during translocation to the membranes (27) or cytoskeleton (16) and also in a kinase-activating cell-free system (28). Green et al. (29) have reported that priming of NADPH oxidase by interleukin 8, another proinflammatory cytokine, does not induce translocation of cytosolic oxidase components. However, DeLeo et al. (11) recently reported that priming of the respiratory burst by lipopolysaccharide resulted in translocation of cytochrome b and p47phox but not p67phox or rac2. The mechanisms underlying lipopolysaccharide priming could be different from those underlying GM-CSF and IL-8 priming.

Our observation that GM-CSF induced partial p47phox phosphorylation but not p67phox phosphorylation supports the idea that p47phox phosphorylation on critical sites plays a major role in the priming of NADPH oxidase in intact cells. The position of the phosphorylated peptides on the phosphopeptide map of p47phox (24) suggests that candidate targets for GM-CSF-induced phosphorylation are serines 328, 345, 348, 359, and 370, contrary to serines 303, 304, 320, and 315.

The GM-CSF receptor is not coupled to classical G proteins. Engagement of GM-CSF with its receptor activates a number of signal transduction pathways, including protein-tyrosine kinase (30), PLCδ1 (31), phospholipase A2 (32), and mitogen-activated protein kinase (ERK1/ERK2) (33, 34). Our co-inhibition experiments suggest that a genistein-sensitive protein-tyrosine kinase and PLCδ1 are involved in GM-CSF-induced partial p47phox phosphorylation and in NADPH oxidase priming. Genistein and wortmannin also inhibited GM-CSF priming of the respiratory burst in response to fMLP in parallel to p47phox phosphorylation (data not shown). In addition, Kodama et al.
(35) recently described a close correlation between the inhibitory effect of wortmannin on PI3K activity, superoxide dismutase (SOD), and p47phox phosphorylation in GM-CSF-primed and fMLP-treated neutrophils; their results support our hypothesis. The GM-CSF receptor interacts with lyn, a member of the src-family nonreceptor tyrosine kinase family, and PI3K can be directly regulated by lyn (31, 36). Because we only detected phosphorylated serines on p47phox, these data suggest that GM-CSF activates Ser/Thr-protein kinase(s) that phosphorylate(s) p47phox downstream of lyn and PI3K. In addition, G109203X-sensitive protein kinase C isoforms are not involved in this process.

In conclusion, the data presented here show that priming of the neutrophil respiratory burst by GM-CSF results in partial p47phox phosphorylation, which increases the degree of p47phox phosphorylation and NADPH oxidase assembly in response to a secondary applied stimulus such as fMLP. This GM-CSF priming pathway involves one or more protein-tyrosine kinases and PI3K. Identification of the Ser/Thr kinase involved in p47phox phosphorylation will require further investigations.

Acknowledgments—We thank Dr. B. M. Babior from the Scripps Research Institute (La Jolla, CA) for the anti-p47phox antibody, Dr. J. J. Stiehl (Scripps Research Institute, La Jolla, CA) for the anti-p47phox antibody, Dr. F. Morel (GREPI, JE-UJF, CHU Grenoble, France) for the anti-p47phox antibody. We also thank V. Ollivier, S. Martin, O. Cachia, and F. Breton from our laboratory for their help.

REFERENCES
1. Babior, B. M. (1984) Blood 64, 959–966
2. Chanock, S. J., El Benna, J., Smith, R. M., and Babior, B. M. (1994) J. Biol. Chem. 269, 24519–24522
3. Wientjes, F. B., and Segal, A. W. (1995) Semin. Cell Biol. 6, 357–365
4. Morel, F., Deussiere, J., and Vignais, P. V. (1991) Eur. J. Biochem. 201, 523–546
5. Downey, G. P., Fukushima, T., Fialkow, L., and Waddell, T. K. (1995) Semin. Cell Biol. 6, 345–356
6. Hallett, M. B., and Lloyds, D. L. (1995) Immunol. Today 16, 264–268
7. Elhim, C., Bailly, S., Chollet-Martin, S., Hakim, J., and Gougerot-Pocidalo, M. A. (1993) Blood 82, 633–640
8. Elhim, C., Bailly, S., Chollet-Martin, S., Hakim, J., and Gougerot-Pocidalo, M. A. (1994) Infect. Immun. 62, 2195–2201
9. Atkinson, YH., Marasco, W. A., Lopez, A. F., and Vadas, M. A. (1988) J. Clin. Invest. 81, 759–765
10. Mc Coll, S. R., Beausigle, D., Gilbert, C., and Naccache, P. H. (1990) J. Immunol. 145, 3047–3053
11. DeLeo, F. R., Renee, J., McCormick, S., Nakamura, M., Apicella, M., Weiss, J. P., and Nauseef, W. M. (1998) J. Clin. Invest. 101, 455–463
12. Clark, R. A., Volpp, B. D., Leidal, K. G., and Nauseef, W. M. (1990) J. Clin. Invest. 85, 714–721
13. Wientjes, F. B., Heenan, J. J., Totty, N. F., and Segal, A. W. (1993) Biochem. J. 301, 557–561
14. Quinn, M. T., Evans, T., Loetterle, I. R., Jessaitis, A. J., and Bokoch, G. M. (1993) J. Biol. Chem. 268, 20883–20887
15. Abo, A., Webb, M. R., Grogan, A., and Segal, A. W. (1994) Biochem. J. 298, 585–591
16. El Benna, J., Ruedi, J. M., and Babior, B. M. (1994) J. Biol. Chem. 269, 6729–6734
17. Okamura, N., Curnutte, J. T., Roberts, R. L., and Babior B. M. (1988) J. Biol. Chem. 263, 6677–6682
18. Dusi, S., and Rossi, P. (1993) Biochem. J. 296, 367–371
19. El Benna, J., Dang, P. M. C., Gaudry, M., Fay, M., Morel, F., Hakim, J., and Gougerot-Pocidalo M. A. (1997) J. Biol. Chem. 272, 17204–17208
20. Nauseef, W. M., Volpp, B. D., McCormick, S., Leidal, K. G., and Clark, R. A. (1991) J. Biol. Chem. 266, 5911–5917
21. Ding, J., Vlahos, C. J., Liu, R., Brown, R. F., and Badway, J. A. (1995) J. Biol. Chem. 270, 11604–11609
22. Faust, L. P., El Benna, J., Babior, B. M. and Chanock, S. J. (1995) J. Clin. Invest. 96, 1499–1505
23. El Benna, J., Faust, L. P., and Babior, B. M. (1994) J. Biol. Chem. 269, 23431–23436
24. El Benna, J., Faust, L. P., Johnson, J. L., and Babior, B. M. (1996) J. Biol. Chem. 271, 6374–6378
25. Laemmli, U. K. (1970) Nature 227, 680–685
26. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
27. Rotrosen, D., and Leto, T. L. (1990) J. Biol. Chem. 265, 2681–2690
28. Park, J. W., Hoyal, C. R., El Benna, J., and Babior, B. M. (1997) J. Biol. Chem. 272, 11035–11043
29. Green, S. P., Chughtarapapai, A., and Curnutte, J. T. (1996) J. Biol. Chem. 271, 25400–25405
30. Gaudry, M., Gilbert, C., Barabé, F., Poubelle, P. E., and Naccache, P. (1995) Blood 86, 3567–3574
31. Corey, S., Eguna, A., Puyana-Theall, K., Bolen, J. B., Cantley, L., Mollinedo, F., Jackson, T. R., Hawkins P. T., and Stephens, L. L. (1995) EMBO J. 14, 2681–2690
32. Durst, M., Durst, S., Molski, T. F. P, Becker, E. L., and Sha'Afi, R. I. (1994) EMBO J. 12, 2681–2690
33. Gomez-Cambronero, J., Huang, C. K., Gomez-Cambronero, T. M., Waterman, W. H., Becker, E. L., and Sha'Afi, R. I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7551–7555
34. Thompson, H. L., Marshall, C. J., and Saklatvala, J. (1994) J. Biol. Chem. 269, 9486–9492
35. Kodama, T., Hazei, K., Hazei, O., Okada, T., and Ui, M. (1999) Biochem. J. 345, 261–269
36. Ptacsnik, A., Prosnitz, E. R., Yoshikawa, D., Smrcka, A., Traynor-Kaplan, A. E., and Bokoch, G. M. (1996) J. Biol. Chem. 271, 25204–25207