Isolation and Characterization of a Variant HL60 Cell Line Defective in the Activation of the NADPH Oxidase by Phorbol Myristate Acetate

Marianne Tardif, Marie-Josèphe Rabiet, Thierry Christophe, Marie-Danielle Milcent and François Boulay

J Immunol 1998; 161:6885-6895; ;
http://www.jimmunol.org/content/161/12/6885

References
This article cites 66 articles, 43 of which you can access for free at:
http://www.jimmunol.org/content/161/12/6885.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Isolation and Characterization of a Variant HL60 Cell Line Defective in the Activation of the NADPH Oxidase by Phorbol Myristate Acetate\textsuperscript{1}

Marianne Tardif,\textsuperscript{2} Marie-Josèphe Rabiet, Thierry Christophe, Marie-Danielle Milcent, and François Boulay

Promyelocytic human leukemia HL60 cells can be differentiated into neutrophil-like cells that exhibit an NADPH oxidase activity through direct stimulation of protein kinase C (PKC) with PMA or through formyl peptide receptor activation. We have isolated a variant HL60 clone that exhibited a conditional PMA-induced oxidative response depending on the agent used for the differentiation. While cells differentiated with DMSO responded to either PMA or \textit{N}-formyl peptide (\textit{N}-formyl-Met-Leu-Phc-Lys or fMLFK), cells differentiated with dibutyryl-cAMP (Bt\textsubscript{c}AMP) responded to fMLFK but very poorly to PMA. However, in Bt\textsubscript{c}AMP-differentiated cells, the expression of the different PKC isoforms was similar to that observed in DMSO-differentiated cells. Moreover, PMA was able to induce a normal phosphorylation of the cytosolic factor p47\textsubscript{phox} and to fully activate extracellular signal-regulated kinases (Erk1/2). Interestingly, Bt\textsubscript{c}AMP-differentiated cells exhibited a strong and sustained O\textsubscript{2}\textsuperscript{–} production when costimulated with PMA and suboptimal concentrations of fMLFK which were, per se, ineffective. This sustained response was only slightly reduced by the conjunction of the mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor PD98059 and wortmannin, a phosphatidylinositol-3 kinase (PI3K) inhibitor. Variant HL60 cells that were stably transfected with a constitutively active form of Rac1 were able, when differentiated with Bt\textsubscript{c}AMP, to secrete oxidant following PMA stimulation. Altogether, the results suggest that, in addition to the phosphorylation of p47\textsubscript{phox}, the activation of NADPH oxidase requires the activation of a Rac protein through a pathway that diverges at a point upstream of MEK and that is independent of the activation of wortmannin sensitive PI3K. \textit{The Journal of Immunology, 1998, 161: 6885–6895.}

\textsuperscript{1} This work was supported by grants from the Centre National de la Recherche Scientifique, Commissariat à l’Énergie Atomique (CEA)/Grenoble, Laboratoire de Biochimie et de Biophysique des Systèmes Intégrés (UMR 314 CEA/Centre National de la Recherche Scientifique), Grenoble, France.

\textsuperscript{2} Address correspondence and reprint requests to Dr. Marianne Tardif, Département de Biologie Moléculaire et Structurale/Biochimie et Biophysique des Systèmes Intégrés (UMR 314 CEA/CNRS), Centre d’Études Nucléaires de Grenoble (CENG), 17 rue des Martyrs, 38054 Grenoble Cedex 9, France.

\textsuperscript{3} Abbreviations used in this paper: PKC, protein kinase C; PI3K, phosphatidylinositol-3 kinase; fMLFK, \textit{N}-formyl-Met-Leu-Phe-Lys; Bt\textsubscript{c}AMP, dibutyryl cyclic AMP; PTX, Bordetella pertussis toxin; WT, wortmannin; MBP, myelin basic protein; Erk, extracellular signal regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen extracellular-regulated kinase; SAPK, stress-activated protein kinase.
intact cell oxidase activation (22, 23). The addition of chemota-
tractants to neutrophils results in the exchange of GTP for GDP on
Rac and in the translocation of this latter to the plasma membrane
(24, 25). However, while the translocation of p67phox and p47phox
kinetically parallels NADPH oxidase activity, the extent of trans-
location of Rac2 from the cytosol to the plasma membrane does
not correlate with neutrophil NADPH oxidase activity, suggesting
that the Rac proteins do not regulate NADPH oxidase activity stoichiometrically (26).

Several studies have demonstrated that the exposure of neutro-
phils to N-formyl peptides increases phosphatidylinositol-3 kinase
(PI3K) activity (27, 28) and p42/44 mitogen-activated protein ki-
nase (MAPK) (or Erk1/2) activities (29–31). The activation of these enzymes was timely correlated with the triggering of
NADPH oxidase. Because PI3K inhibitors such as wortmannin
(WT) and LY294002 completely abolished the superoxide secre-
tion induced by N-formyl peptides but not that mediated by PMA
(32–37), it has been suggested that PI3K is a key component, up-
stream of PKC, in the transduction pathway involved in chemota-
tractant-mediated NADPH oxidase activation. However, the role of the p42/44 MAPKs is less clear. On the one hand, a functional
link with NADPH oxidase activity is supported by the observation that,
among the serine residues of p47phox that are phosphorylated
upon stimulation, two are located within consensus sequences for
proline-directed kinases (10) and correspond to the sites phosphor-
ylated by Erk2 in vitro (11, 38). Moreover, NADPH oxidase has been
found to be inhibited by PD98059, a compound that specific-
ally inhibits the activation of MEK, the upstream kinase of Erk
(39–42). On the other hand, recent studies suggest that even
though both chemoattractant- and PMA-mediated MAPK activa-
tions coincide with superoxide generation, MAPK and oxidase ac-
tivities are not functionally correlated (43–46). Several other stud-
ies point out that the p38 stress-activated protein kinase (SAPK) is
a better candidate for the regulation of superoxide generation by
neutrophils (38, 41, 47–50).

In the present study, we examined a variant clone of the pro-
myelocytic cell line HL60 for which a PMA-dependent superoxide
production could be elicited in DMSO-differentiated cells but only
poorly in Bt2cAMP-differentiated cells. In these latter cells, a robust and sustained production of superoxide was however ob-
erved when stimulatory (i.e., nanomolar) concentrations of N-
formyl peptide were used in association with PMA. This synergistic response was somewhat reminiscent of the numerous patterns of priming described in neutrophils, where nonstimulatory concentrations of chemoattractant (1–10 nM) potentiated the ox-
idase response initiated by another agonist (1). This variant HL60
cloned appeared to be a suitable tool to delineate the mechanisms of oxidase activation. We examined the status of the already known
pathways leading to NADPH oxidase activation (p47phox phos-
phorylation, Rac, PI3K, and MAPK activations). Our results showed that in addition to the phosphorylation of p47phox, the
activation of NADPH oxidase requires a second pathway which most
probably involves the activation of Rac in a PI3K and MEK-in-
dependent manner.

**Materials and Methods**

**Reagents**

PMA, WT, N-formyl-Met-Leu-Pho-Lys (fMLFK), N-O-2'-dibutylryl cy-
colic AMP (Bt2cAMP), DMSO, BSA, myelin basic protein (MBP), 2-ME,
leupeptin, benzamidine, pepstatin, aprotinin, PMSF, p-nitrophenylphos-
phate, pertussis toxin (PTX), cytochrome C, anti-mouse IgG were obtained
from Sigma (St. Louis, MO). 4-(2-aminoethyl)-benzenesulfonyl fluoride
hydrochloride (AEBSP) was from Boehringer Mannheim (Indianapolis,
IN). PD98059 was purchased from Calbiochem-Novabiochem (San Diego,
CA). Protein A-Sepharose and protein G-Sepharose 4 fast flow were pur-
ched from Pharmacia Biotech (Uppsala, Sweden). Peroxidase-conju-
gated protein A was from Bio-Rad (Irvine, France). Anti-rabbit IgGs per-
oxidase linked Abs and enhanced chemiluminescence Western blotting
detection system (ECL) were purchased from Amersham (Les Ulis, France).
Anti-Rac1 polyclonal Ab (sc-217), anti-RhoGDI polyclonal Ab
(sc-360), and polyclonal Abs against MAPKs Erk1 and -2 (sc-93 and sc-
103) were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Abs
against PKC isozymes were purchased from Transduction Laboratory
(Lexington, KY). Ab against p47phox was raised against the C-terminal
peptide (NH2-Y-E-S-T-K-R-K-L-A-S-A-V-OH) that was synthesized by BioCytex
(Marseille, France). The anti-c-myc mouse mAb (9E10) and competing c-myc
peptide were purchased from Boehringer Mannheim. Cell culture medium,
FCS, and geneticin (G418) were from Life Technologies (Grand Island, NY), except for phosphate-free RPMI which was provided by
ICN. (Orsay, France) [32P]Orthophosphoric acid was purchased from
Isotopohim (Ganagobie, France). Redivue Pro-mix L-[35S] in vitro cell label-
ing mix was obtained from Amersham.

**Cell culture and differentiation**

Promyelocytic HL60 cells were cultured in RPMI 1640-glutaMAX I me-
dium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and
10% of heat inactivated FCS at 37°C in humidified atmosphere of 5% CO2/95% air. Cell differentiation was initiated either with 1.25% DMSO
for 6 days when cell density was 0.5 × 10⁷ cells/ml or with 1 μM
Bt2cAMP for 3 days. In this latter case, optimal differentiation was ob-
tained when cells were centrifuged and resuspended at a density of 10⁶
cells/ml in fresh complete RPMI before differentiation was initiated.

**Isolation of a variant HL60 clone**

Promyelocytic HL60 cells were diluted at a density of about 100 cells for
10 ml of conditioned RPMI filtered through a 0.2-μm sterile Millipore
(Bedford, MA) filter. Before plating in tissue culture plates, the cell sus-
pension was maintained at 37°C and supplemented with sterile agarose at
the final concentration of 0.18%. After 2 wk, clones were picked with a Pasteur pipette, expanded in complete RPMI, and tested for the absence of
PMA-induced superoxide production after differentiation with Bt2cAMP.

**Superoxide production assay**

Cells were washed with PBS and resuspended at a concentration of 4 × 10⁷
cells/ml in PBS containing 0.5 mM CaCl₂, 1 mM MgCl₂, and 30 mM
buffer (buffer A). Preincubation with various inhibitors was accomplished
at 37°C for the indicated periods of time. Control cells without inhibitors
were incubated in the same conditions. Fifty microliters of the cell sus-
pension, kept at 4°C, were added to 1 ml of prewarmed PBS containing 200
μM of ferricytochrome C. Ferricytochrome C formation was initiated by the ad-
dition of fMLFK or PMA at the indicated concentrations. Ferricytochrome
C reduction was continuously monitored at 550 nm.

**Cell lysis, subcellular fractionation, and Western blot analysis of
PKC isoforms, RhoGDI, and Rac G proteins**

Cells were washed with PBS and resuspended at a concentration of 2 × 10⁷
cells/ml in buffer A. After stimulation with PMA (1 μg/ml) for 3 min, cells
were harvested and resuspended into 50 mM Tris-HCl (pH 7.5) containing
2 mM EGTA, 50 mM 2-ME, 10 μg/ml leupeptin, 10 mM benzamidine, 5
μM pepstatin, 0.2 μg/ml aprotinin, and 1 mM PMSF. Cells were disrupted
by sonication, and the resulting homogenate was centrifuged (800 × g for
5 min at 4°C) to remove unbroken cells and nuclei. The cytosolic fraction
obtained by centrifugation at 100,000 × g for 1 h at 4°C was supplemented
with Laemmli sample buffer containing 5 mM DTT. The remaining pellet
was solubilized by sonication in Laemmli sample buffer containing 5 mM
DTT (51). Samples were denatured by boiling for 5 min, subjected to
electrophoresis on a 10% SDS-polyacrylamide gel, and electrotransferred
to nitrocellulose. Nonspecific binding was blocked by incubation of the
membrane with PBS containing 0.1% Tween-20 (PBS-T) and 3% BSA for
1 h at room temperature. The membrane was then incubated overnight
at 4°C with appropriate dilutions (1:250 to 1:1000) of mAbs to the various
PKC isoforms in PBS-T containing 3% BSA. After washing in PBS-T, the
membrane was incubated with a 1:100 dilution of rabbit anti-mouse IgG
(Sigma) in PBS-T containing 1% BSA for 2 h. After washing of the mem-
brane in PBS-T, detection of PKC isozymes was performed using 125I-
labelled protein A and autoradiography on Fuji RX films (Fuji Medical
Systeme, Clichy, France).

RhoGDI and Rac1 polyclonal Abs were used at the dilution 1:100. Bound RhoGDI IgGs were detected with 125I-labelled protein A whereas
bound Rac1 IgGs were visualized with anti-rabbit IgGs peroxidase-linked
Abs and ECL.
Immunoprecipitation of MAPKs

Differentiated HL60 cells were resuspended in buffer A (10^7 cells/ml) and stimulated either with FMLPK (10 nM) or PMA alone (1 µg/ml) or with the combination of both (10 nM FMLPK and 10 nM PMA) for a total time of 3 min. In the latter case, FMLPK was added 1 min after PMA. Stimulation was terminated by a brief centrifugation and lysis of the cell pellet at 4°C in 800 µl of lysis buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM sodium phosphate, 10 mM NaF, 1% Triton X-100, 0.5% m-nitrovanadate, and antiproteases (10). In brief, the excised band was soaked in 200 µl of electrophoresis buffer (formic acid/acetic acid/water, 1:3:36, v/v). Detection of 32P-labeled peptides was performed through PhosphorImager.

Expression of the constitutively active Rac1 in the variant HL60 clone

The pE-fmyc-tagged Rac1V12 expression plasmid was constructed as follows. The pMT90 plasmid kindly provided by Dr. P. Chavrier (Marseille, France) was digested with NotI and BamHI. The resulting pE-fmyc-tagged Rac1V12 containing fragment was cloned between the NotI and BamHI sites of the pCNDA3.1 (In Vitrogen, Groningen, The Netherlands). The resulting plasmid was digested with PstI and the fragment containing the pE-fmyc-tagged Rac1V12 sequence was cloned into the Smal site of a modified version of the pE-neo plasmid kindly provided by Dr. M. C. Dinauer (Indianapolis, IN) (53).

Transfection of the variant HL60 clone was performed by electroporation with a Bio-Rad Gene Pulser apparatus, according to Tonetti et al. (54) with slight modifications. In brief, 20 µg of supercoiled plasmid DNA in TE (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)) were mixed with 10^7 cells in 0.5 ml of phosphate-buffered sucrose (272 mM sucrose and 7 mM Na3HPO4 (pH 7.4)). Cells were electroporated with a pulse of 250 V for 18–20 ms. Control cells were transfected in the same conditions with the pE-neo plasmid. Following electroporation, cells were allowed to recover in 100 µl of culture medium at 37°C for 48 h before selection with 0.6 mg of G418/ml of medium. G418-resistant transfected clones were obtained by limited dilution into 24-well microtiter plates.

The presence of the pE-fmyc-tagged Rac1V12 mRNA in the G418-resistant clones was detected by a reverse transcriptase PCR assay of DNase-treated RNA. Poly(A) + mRNA was isolated from 5 × 10^7 cells samples using the Strataclean kit (Stratagene) mRNA isolation system (Novagen, Madison, WI). First strand DNA synthesis was performed using random hexamers as primers and Expand Reverse Transcriptase (Boehringer Mannheim). Fragments corresponding to myc-Rac1V12 and endogenous Rac1 cDNAs were amplified by PCR with the following primers: 5’-CTCATCTCAGAAGAGGATCTGCTG3’ (sense in the tag sequence), 5’-TGCCCAAGTTATGATAGATG3’ (sense in Rac1 sequence), and 5’-AGAAGCTCTCGAGCGATGATGCT3’ (antisense in Rac1 sequence). The products were resolved on a 1% agarose gel and stained with ethidium bromide.

For detection of myc-tagged Rac1V12 protein, 5 × 10^5 HL60 cells transfected with either pE-fmyc-Rac1V12 or pE-fneomio-plasmid were washed twice with 10 ml of methionine/cystine-deficient RPMI and cultured for 60 min in this culture medium. Cells at the density of 10^6 cells/ml were labeled by the addition of 3.75 µCi of Redivue Pro-mix L-[35S] in vitro cell labeling mix for 3 h. Cells were then washed once with 10 ml of complete RPMI and cultured for another hour in 10 ml of complete RPMI. Cell pellets were lysed in 1 ml of ice-cold RIPA buffer supplemented with protease inhibitors. Lysates were centrifuged for 5 min at 14,000 × g, and supernatants were subjected to immunoprecipitation with 10 µg of anti-c-myc mAb (9E10) coupled to protein-G Sepharose beads for 1 h. The Sepharose beads were subjected to four washes with 1 ml of ice-cold RIPA buffer. Sepharose beads were treated with 80 µl of 2-fold Laemmli sample buffer with reducing agents for 5 min at 100°C, and loaded on a 14% SDS-polyacrylamide gel. After fixation and incubation in 1 M sodium sallycylate in 20% methanol, the gel was dried and exposed to x-ray film.

Results

Isolation and oxidase phenotype of the variant HL60 cell line

Wild-type HL60 cells from the American Type Culture Collection (Manassas, VA) can be differentiated to granulocyte-like cells with DMSO or Bt2-CAMP. They similarly produce superoxide in response to PMA or N-formyl peptide (iMLFK) whatever the differentiation protocol used. While testing a new FCS, we selected a rapidly growing population of cells that proved to respond very poorly to PMA when differentiated with Bt2-CAMP. A clone was further isolated on soft agar and characterized. When assayed for NADPH oxidase activity after differentiation with DMSO, the isolated variant HL60 cells exhibited a rapid and strong response to both N-formyl peptide and PMA (Fig. 1A, traces d and e). However, after differentiation with Bt2-CAMP, the variant HL60 cells responded to a saturating concentration of N-formyl peptide (1 µM) but virtually failed to respond to PMA (1 µg/ml), even after a prolonged exposure (Fig. 1A, traces a and b, respectively). PMA was nevertheless able to induce a strong and sustained superoxide chromophor in n-butanol/pyridine/acetate acid/water (75:50:15:60, v/v).

Downloaded from http://www.jimmunol.org/ by guest on April 16, 2017
production in Bt2cAMP-differentiated cells when used in conjunc-
tion with a suboptimal concentration of fMLFK (10 nM) which
failed to activate the NADPH oxidase (Fig. 1A, traces b and c).
This sustained response was observed independently of the order
of addition of the two agonists.

The possibility to evoke oxidative activity in Bt2cAMP-differ-
entiated variant HL60 cells indicates that components of the
NADPH oxidase complex were structurally functional. Moreover,
the response elicited by the combination of the two activators was
persistent over at least 10 min, while the response elicited by 1
mM of N-formyl peptide alone was rapidly attenuated. To compare
the ability of fMLFK to stimulate per se NADPH oxidase activity and
its potency to rescue the PMA response, we quantified the amount
of superoxide produced by Bt2cAMP-differentiated cells stimu-
lated with varying concentrations of fMLFK in the absence or

FIGURE 1. Oxidase activity in differentiated variant HL60 cells. A. Oxidase assay was performed on variant HL60 cells induced with Bt2cAMP for 3 days (traces a–c) or with DMSO for 6 days (traces d and e). Kinetics of superoxide production were recorded using the cytochrome c reduction assay as described in Materials and Methods. fMLFK (1 μM or 10 nM as indicated) or PMA (1 μg/ml) was added in the cuvette at the time indicated by the arrows. Numbers on the left side of each trace indicate the initial rate of superoxide production (nmol/min/10⁶ cells). B, fMLFK dose responses of oxidase activation. Variant HL60 cells differentiated with Bt2cAMP were incubated in the absence (open circles) or the presence (filled circles) of PMA (1 μg/ml) for 2 min before stimulation with increasing concentrations of fMLFK. The amount of superoxide produced after 5-min stim-
ulation was reported as percentage of the maximal response, obtained with 1 μM fMLFK in the presence of PMA. Values represent the means ± SD of four independent experiments.

FIGURE 2. Effect of PTX on the oxidase response in Bt2cAMP-differ-
entiated variant HL60 cells. Bt2cAMP-differentiated cells were cultured for
5 h in the absence or the presence of 100 ng PTX/ml before cytochrome c re-
duction assay. A, Cytochrome c reduction kinetics upon stimulation with either 1 μM fMLFK or PMA plus 10 nM fMLFK. B, The total amount of superoxide produced after 6-min stimulation as in A was measured. For each set of agonists, the amount of superoxide produced by PTX-treated cells was expressed as the percentage of the amount obtained with control
values represent the means ± SD of three independent experiments.

As expected, the NADPH oxidase stimulation in response to a
saturating concentration of fMLFK alone was fully inhibited by
pretreating cells for 5 h in the presence of 100 ng/ml PTX. How-
ever, the response triggered by the combination of PMA plus 10
nM fMLFK was never completely inhibited. About 10–15% of the
response was reproducibly resistant to PTX treatment (Fig. 2, A
and B). This suggests that although a Gi-type heterotrimeric G
protein, probably Gi2, is mainly involved in signal transduction
through chemoattractant receptor in differentiated HL60 cells, a
coupling of the N-formyl peptide receptor to PTX-resistant het-
rotetramer G protein(s) may exist in these cells. A residual amount
of PTX-resistant Ga16 may still be present, although its expres-
sion has been shown to dramatically decrease during the course of
differentiation to granulocytes (55).

Phosphorylation of p47phox in the variant HL60 cell line
Phosphorylation of the cytosolic factor, p47phox, is thought to be a
key step in PMA- and agonist-mediated activation of the NADPH
oxidase. We therefore examined whether under PMA stimulation

production in Bt2cAMP-differentiated cells when used in conjunc-
tion with a suboptimal concentration of fMLFK (10 nM) which
failed to activate the NADPH oxidase (Fig. 1A, traces b and c).
This sustained response was observed independently of the order
of addition of the two agonists.

The possibility to evoke oxidative activity in Bt2cAMP-differ-
entiated variant HL60 cells indicates that components of the
NADPH oxidase complex were structurally functional. Moreover,
the response elicited by the combination of the two activators was
persistent over at least 10 min, while the response elicited by 1 μM
of N-formyl peptide alone was rapidly attenuated. To compare
the ability of fMLFK to stimulate per se NADPH oxidase activity and
its potency to rescue the PMA response, we quantified the amount
of superoxide produced by Bt2cAMP-differentiated cells stimu-
lated with varying concentrations of fMLFK in the absence or

presence of PMA, respectively. When PMA was present, super-
oxide production was detectable with concentrations of fMLFK as
low as 100–200 pM (Fig. 1B). A plateau was reached at ~10 nM
and the concentration for a half-maximal restoration of the PMA
response was about 3 nM. In the absence of PMA, superoxide
production was detectable only for concentrations superior
to 20 nM-50 nM fMLFK.

The rescue of PMA by N-formyl peptide is partially PTX-
resistant
As expected, the NADPH oxidase stimulation in response to a
saturating concentration of fMLFK alone was fully inhibited by
pretreating cells for 5 h in the presence of 100 ng/ml PTX. How-
ever, the response triggered by the combination of PMA plus 10
nM fMLFK was never completely inhibited. About 10–15% of the
response was reproducibly resistant to PTX treatment (Fig. 2, A
and B). This suggests that although a Gi-type heterotrimeric G
protein, probably Gi2, is mainly involved in signal transduction
through chemoattractant receptor in differentiated HL60 cells, a
coupling of the N-formyl peptide receptor to PTX-resistant het-
rotetramer G protein(s) may exist in these cells. A residual amount
of PTX-resistant Ga16 may still be present, although its expres-
sion has been shown to dramatically decrease during the course of
differentiation to granulocytes (55).

Phosphorylation of p47phox in the variant HL60 cell line
Phosphorylation of the cytosolic factor, p47phox, is thought to be a
key step in PMA- and agonist-mediated activation of the NADPH
oxidase. We therefore examined whether under PMA stimulation

FIGURE 2. Effect of PTX on the oxidase response in Bt2cAMP-differ-
entiated variant HL60 cells. Bt2cAMP-differentiated cells were cultured for
5 h in the absence or the presence of 100 ng PTX/ml before cytochrome c re-
duction assay. A, Cytochrome c reduction kinetics upon stimulation with either 1 μM fMLFK or PMA plus 10 nM fMLFK. B, The total amount of superoxide produced after 6-min stimulation as in A was measured. For each set of agonists, the amount of superoxide produced by PTX-treated cells was expressed as the percentage of the amount obtained with control
values represent the means ± SD of three independent experiments.

FIGURE 1. Oxidase activity in differentiated variant HL60 cells. A. Oxidase assay was performed on variant HL60 cells induced with Bt2cAMP for 3 days (traces a–c) or with DMSO for 6 days (traces d and e). Kinetics of superoxide production were recorded using the cytochrome c reduction assay as described in Materials and Methods. fMLFK (1 μM or 10 nM as indicated) or PMA (1 μg/ml) was added in the cuvette at the time indicated by the arrows. Numbers on the left side of each trace indicate the initial rate of superoxide production (nmol/min/10⁶ cells). B, fMLFK dose responses of oxidase activation. Variant HL60 cells differentiated with Bt2cAMP were incubated in the absence (open circles) or the presence (filled circles) of PMA (1 μg/ml) for 2 min before stimulation with increasing concentrations of fMLFK. The amount of superoxide produced after 5-min stim-
ulation was reported as percentage of the maximal response, obtained with 1 μM fMLFK in the presence of PMA. Values represent the means ± SD of four independent experiments.
p47\textsuperscript{phox} was differentially phosphorylated in cells differentiated with DMSO or Bt2cAMP. After metabolic labeling of cells with \textsuperscript{32}Porthophosphate and stimulation, p47\textsuperscript{phox} was isolated by immunoprecipitation and submitted to SDS-PAGE. Consistent with the fact that about three times as much p47\textsuperscript{phox} was expressed in DMSO-differentiated cells (2 \times 10\textsuperscript{7} cells) that were not stimulated (lane 1) or exposed to PMA for 2 min (lane 2), Bt2cAMP-differentiated cells (2 \times 10\textsuperscript{7} cells) that were not stimulated (lane 3) or exposed to PMA for 2 min (lane 4) or to PMA for 1 min, then to 10 nM fMLFK for another min (lane 5). Immunoprecipitated material was separated on a 10% SDS-polyacrylamide gel under reducing conditions and visualized with a PhosphorImager. C, Tryptic maps of \textsuperscript{32}P-labeled p47\textsuperscript{phox}. Stimulation of \textsuperscript{32}P-prelabeled differentiated cells were as in B, except that in the case of Bt2cAMP-differentiated cells twice as much cells were used (4 \times 10\textsuperscript{7} vs 2 \times 10\textsuperscript{7} DMSO-differentiated cells). After SDS-PAGE and transfer onto nitrocellulose sheets, the bands corresponding to immunoprecipitated p47\textsuperscript{phox} were excised and digested with trypsin. Mapping of the resulting phosphopeptides was conducted as described in Materials and Methods.

Expression of PKC isoforms in differentiated variant HL60 cells

Phorbol esters are direct activators of a number of PKCs. Upon cell activation with PMA, translocation of PKC to the cell membrane has been observed and is associated with the activation of the enzyme (56). To examine whether the failure of PMA to activate the NADPH oxidase in Bt2cAMP-differentiated cells could arise...
from a deficiency in the expression or function of one PKC isoform, we analyzed the distribution of the various isoforms in subcellular fractions of DMSO- and Bt2cAMP-differentiated HL60 cells using specific Abs. In either type of differentiated cells, isoforms α, β, γ, δ, ε, η, λ, and ζ were detected (Fig. 4). They corresponded to the isoforms previously detected in neutrophils (8, 12, 57) or in granulocyte-type differentiated HL60 cells (58, 59). PKC η, θ, and μ were not visible whether cells were differentiated with DMSO or Bt2cAMP (data not shown). In nonstimulated cells, PKC α, β, and γ were detected predominantly in the cytosolic fraction, whereas PKC δ and ε were mainly recovered in the particulate membrane fraction. Distribution of PKC isoforms ζ, λ, and ι were almost equally distributed between the cytosolic and the particulate membrane fraction. Treatment of cells with PMA induced a substantial translocation of cytosolic PKC α, β, γ, and δ to the particulate membrane fraction, indicating their activation upon PMA. Distribution of PKC ε, ζ, λ, and ι appeared to be unchanged after PMA stimulation whichever conditions were used for the differentiation. The atypical isoforms λ, ι, and ζ, which have been shown to be unaffected by phorbol esters (56), were not expected to be translocated. The distribution of the most easily detectable atypical isoform (i.e., PKC ζ) was not modified even when cells were costimulated with PMA and a low concentration of N-formyl peptide. Altogether, the results indicate that there was no obvious abnormality in the PKC isoforms expression and distribution in Bt2cAMP-differentiated cells.

Effects of WT and PD98059 on the NADPH oxidase and Erks activities in Bt2cAMP-differentiated HL60

In neutrophils, the binding of chemoattractants to their cognate receptors results in activation of PI3K and of Erk1/2 MAPK. As these enzymes were proposed to participate in the induction of the oxidative burst, we examined in Bt2cAMP-differentiated HL60 the effects of PI3K and MEK inhibitors, namely WT and PD98059, on superoxide production and on Erk2 phosphorylating activity elicited under the various conditions of stimulation.

As illustrated in Fig. 5, WT strongly inhibited the response to 1 μM IMLFK (hatched bars). The extent of inhibition obtained at 10 nM and 100 nM WT were of 50% and 80%, respectively. PD98059 at best reduced the same response by 30%. However, treatment of Bt2cAMP-differentiated variant HL60 cells with 10 nM or 100 nM WT or with 50 μM or 100 μM PD98059 had only a slight inhibitory effect (inferior to 20%) on superoxide production when cells were costimulated with PMA plus 10 nM IMLFK (filled bars). These results suggest that, under costimulation conditions, the activation of the NADPH oxidase complex elicited by substimulatory concentrations of chemoattractant proceeds through a pathway in which PI3K and MEK play no major role.

Both the Erk inhibitor and the PI3K inhibitor have been shown to alter MAPK activities in vivo (39–42, 44–46, 60, 61). Their respective effect in Bt2cAMP-differentiated variant cells were thus checked on Erk2 activity as assayed by the ability of Erk2 immune complexes to phosphorylate the MBP in vitro (Fig. 6). Only a very weak basal activity was observed in control cells that have not been stimulated. Phosphorylation of MBP was markedly increased when Erk2 was immunoprecipitated from cells stimulated with 10 nM IMLFK (or 1 μM IMLFK; data not shown) or with PMA alone. The combination of the two agonists resulted in an even stronger Erk2 activity (Fig. 6, first track of last panel). Only partial inhibitions of stimulated Erk2 activity were obtained after a pretreatment of the cells with PD98059. Under stimulation with IMLFK, PMA, or the combination of both, Erk2 activity was reduced by 42 ± 15%, 68 ± 16%, or 27 ± 18% (n = 3), respectively. However, much stronger inhibitory effects were observed while combining PD98059 and WT (Fig. 6, last track in each panel). Fig. 5 shows that, in parallel, the NADPH oxidase response to 1 μM IMLFK was completely obliterated by cotreatment of the

FIGURE 5. Effects of inhibitors PD98059 and WT on superoxide production in Bt2cAMP-differentiated variant HL60. PD98059 and WT, used separately or in combination, were added at the indicated concentration to the oxidase assay medium for 1 h or 10 min, respective before agonist stimulation. Bars represent the amount of superoxide produced for 6 min after stimulation with PMA plus 10 nM IMLFK (filled bars) or with 1 μM IMLFK (hatched bars). For each stimulatory condition, the values ± SD (n = 5) were expressed as percentage of control oxidase activity obtained in the absence of inhibitor.

FIGURE 6. Effects of inhibitors PD98059 and WT on MBP phosphorylating activity of Erk2 isolated from Bt2cAMP-differentiated variant HL60 cells. Erk2 isoform was immunoprecipitated from lysates of Bt2cAMP-differentiated cells that have been stimulated with PMA (1 μg/ml), IMLFK (10 nM), or both agonists for 3 min after incubation without inhibitor or with either 100 μM PD98059 for 1 h, 100 nM WT for 10 min, or a combination of both. The figure is representative of three independent experiments. Western blot analyses were systematically performed to check that the same amount of kinase had been immunoprecipitated (data not shown). The MBP phosphorylation assay was conducted as described in Materials and Methods. Assays were also performed with Erk1. Similar results were obtained although weaker activities were observed (data not shown).
cells with WT and PD98059, whereas the fMLF-K-rescued PMA-dependent response was only slightly inhibited (20% inhibition). Thus, in cells simultaneously challenged with PMA and fMLF, it was possible to reduce Erk2 activity by more than 80% while oxidant generation proved to be virtually unaltered. Although the association of the agonists resulted in a stronger Erk2 activity, the sustained superoxide production does not appear to be correlated with the activation of Erk2. This finding supports the former conclusion that the Erk proteins have little, if any, role in the chemotactant-dependent rescuing activity of NADPH oxidase in presence of PMA.

Constitutively active Rac protein can restore PMA-dependent oxidative response in variant HL60 cells

When we analyzed lysates of differentiated HL60 cells by immunoblotting, Rac1 was detected in the cytosolic fraction as a 23-kDa band whether cells were differentiated with DMSO or Bt2cAMP (Fig. 7). Rac1 protein was not immunodetected in the particulate fraction, and the small G protein Rac2 was detected neither in DMSO- nor in Bt2cAMP-differentiated cells with commercially available Abs (data not shown). As already observed for the expression of p47phox (see Fig. 3A), the level of expression of Rac1 and RhoGDI, a protein that associates with Rac in its GDP-bound form, was lower in cells differentiated with Bt2cAMP than in cells differentiated with DMSO (Fig. 7). The fact that the NADPH oxidase complex is functional under chemotactant stimulation in both conditions of differentiation indicates that the low level of expression of these cytosolic components is not a limiting factor in the activation of the NADPH complex.

To examine whether the reduced PMA-mediated superoxide production in Bt2cAMP-differentiated variant HL60 cells could result from a default in the activation of Rac1, we stably expressed a constitutively active form of Rac1 (Rac1V12) by transfecting cells with the vector pEF-myc-Rac1V12. G418-resistant clones were tested for the expression of myc-Rac1V12. myc-Rac1V12 mRNA was detected by RT-PCR as shown in Fig. 8A. A PCR product of 550 bp corresponding to myc-Rac1V12 mRNA (lane 5) and a PCR product of 410 bp corresponding to endogenous Rac1 mRNA (lane 7) were amplified from pEF-myc-Rac1V12-transfected cells. While the 410-bp product was always detected, the 550-bp PCR product was absent in sample derived from the control cells that were transfected with the empty vector pEF (lane 1). The myc-Rac1V12 protein was undetectable by immunoblotting of the cell lysates with an anti-myc Ab (data not shown). However, a 24- to 25-kDa species was immunoprecipitated from the cytosol of myc-Rac1V12-transfected cells after metabolic labeling with [35S]methionine (Fig. 8B). The identity of this band with myc-Rac1V12 was ascertained by the fact that it was not recovered in lysate of control cells and when competing c-myc peptide was added during the immunoprecipitation. The absence of detection of myc-Rac1V12 protein by direct immunoblotting with anti-myc Abs in clones expressing myc-Rac1V12 mRNA most probably results, as mentioned by others (62), from the fact that high expression levels of the constitutively active form of Rac may cause lethality of the cells.
Clones expressing myc-Rac1V12 were differentiated with Bt2cAMP and tested for oxidase activity under stimulation by PMA. As shown in Fig. 8C, these cells were able to produce superoxide in response to PMA to a similar extent as DMSO-differentiated variant cells. This appeared specifically due to Rac1V12 expression as the clones that were transfected with the empty vector failed to produce superoxide under PMA stimulation when differentiated with Bt2cAMP. This finding suggests that the failure of PMA to activate the NADPH oxidase in the variant HL60 cells is consecutive to a defect in Rac activation.

Discussion

P47\textsuperscript{phox} phosphorylation is not sufficient to induce oxidase activity

We have isolated a variant HL60 cell line that is poorly responsive to PMA with respect to the activation of the NADPH oxidase after differentiation into neutrophil-like cells with Bt2cAMP. However, a normal PMA-mediated superoxide production is observed when cells are differentiated with DMSO. Although PMA is inefficient to trigger NADPH oxidase activation in Bt2cAMP-differentiated cells, it is nevertheless able to induce the phosphorylation of p47\textsuperscript{phox}. Judging from the two-dimensional tryptic phosphopeptide analysis of p47\textsuperscript{phox} immunoprecipitated from both Bt2cAMP- and DMSO-differentiated cells, the inability of PMA to induce superoxide production is unlikely to result from a defect in the phosphorylation of only one or a few serine phosphorylation sites of p47\textsuperscript{phox}.

Thus, our results suggest that the phosphorylation of p47\textsuperscript{phox} is not sufficient to trigger the activation of the NADPH oxidase. This result is consistent with the notion that the levels of p47\textsuperscript{phox} phosphorylation and the rates of superoxide production are not correlated. Based on the observation that p47\textsuperscript{phox} is phosphorylated in cells treated with the protein phosphatase inhibitor calyculin A without any stimulation of NADPH oxidase, it has been suggested that the phosphorylation of discrete residues may negatively regulate the NADPH oxidase (63). Several other studies have underscored that a marked reduction in the phosphorylation of p47\textsuperscript{phox} is not always accompanied by an inhibition of formyl peptide-induced oxidase activity (7, 64). However, in a cell-free system, arguments have been provided for the necessity of p47\textsuperscript{phox} to be phosphorylated to serve as a switch in the first step of oxidase activation (13). Our results do not allow us to conclude on the exact role of the phosphorylation of p47\textsuperscript{phox} in triggering the production of superoxide, but they point out to the requirement of a second signal independent of p47\textsuperscript{phox} phosphorylation.

Absence of PMA-mediated superoxide production in Bt2cAMP-differentiated variant HL60 cells does not result from a defective activation of PKC or MAPK

As phorbol esters are direct activators of a number of PKC isoforms, the inability of PMA to activate oxidase in Bt2cAMP-differentiated variant HL60 cells may be due to a defective expression or a functional alteration of PKC. It has been proposed that the PKC-sensitiv\beta isoforms could be involved in the phosphorylation of p47\textsuperscript{phox} (8). The lack of any obvious difference between DMSO- and Bt2cAMP-differentiated cells in the expression and subcellular localization of the immunoanalyzed PKC isoforms indicates that the poor PMA-induced superoxide production in Bt2cAMP-differentiated cells does not result from a defective expression of any PKC isoform. One cannot conclude on the functionality of the isoforms known not to be translocated upon PMA stimulation (\(\xi, \lambda,\) and \(\iota\)). However, since PMA is able to activate Erk1/2 in Bt2cAMP-differentiated cells and based on recent studies indicating that PKC \(\xi\) is critically involved in the activation of MEK/Erk1/2 pathway (65–67), it is tempting to speculate that the phenotype of the variant HL60 cells does not result from a deficiency in PKC \(\xi\).

Chemoattractants stimulate a second pathway that bypasses PI3K and MEK and is involved in rescuing the activation of NADPH oxidase by PMA

A major observation in this study is that despite the failure of PMA alone to trigger a production of superoxide anions in Bt2cAMP-differentiated cells, its association with nonstimulatory doses of chemoattractant restored a full and sustained NADPH oxidase response. The use of specific inhibitors gave us some hints in deciphering the signaling pathway elicited by stimulatory concentrations of chemoattractants. Interestingly, the oxidative response elicited by the addition of a stimulatory concentration of fMLFK with PMA was not inhibited by either WT, PD98059, or the combination of both inhibitors. Moreover, under costimulation conditions, Erk2 activity was dramatically repressed by the combination of WT and PD98059. These observations suggest that low concentrations of chemoattractant activate a pathway bypassing PI3K, MEK, and the MAPK Erk1/2.

The involvement of Erk1/2 in the activation of NADPH oxidase by saturating concentrations of formyl peptide is still a matter of debate. These MAPKs have been proposed as possible effectors of NADPH oxidase activation, mainly because the activation of both NADPH oxidase and Erk1/2 is inhibited by WT (39, 61) or by PD98059 (39–42). However, the dose-dependent effects of these inhibitors are dissimilar regarding Erks activity or NADPH oxidase activity (44–46) and Erk1/2 activity could be dissociated from the oxidative burst in both human neutrophils and differentiated HL60 cells (43). In this study, the lack of a strong inhibitory effect of PD98059 on the production of superoxide could result from a partial inhibition of Erk2 activity when cells were stimulated by the chemoattractant. Our observation that the MEK activation inhibitor (PD98059) modestly repressed Erk2 activation by formyl peptide while having a stronger effect on the activation by phorbol ester suggests that the activation of Erk2 by formyl peptide is partially independent from MEK. Another possibility is that MEK is differentially inhibited by PD98059 depending on whether MEK is activated by formyl peptide or PMA. It has been recently shown that MEK can be differentially activated (via a Raf-dependent or a Raf-independent pathway) according to the PKC isotype that is activated (67).

Taken together, our results suggest that chemoattractant-mediated activation of NADPH oxidase involves two pathways. The first pathway, which requires high concentrations of chemoattractant, flows through PI3K, whereas the second pathway would diverge at a point upstream of WT-sensitive PI3K and MEK. This second pathway can be activated by low concentrations of chemoattractant but is not sufficient, alone, to elicit oxidant secretion. It is likely that the two pathways actually act in concert when NADPH oxidase is activated by a saturating concentration of chemoattractant (see Fig. 8). In the variant HL60 cells differentiated with Bt2cAMP, PMA appears to place the complex in a primed state awaiting further stimulus before the NADPH oxidase response is elicited. The aforementioned PI3K and MEK-independent pathway provides the switch to turn on the oxidase complex. The extremely low concentrations of chemoattractant that are required to turn on this “oxidase switch” suggest that the second pathway is similar to that involved in the chemotaxis of phagocytic leukocytes. Noticeably, some authors using the MEK/Erk inhibitor PD98059 failed to inhibit granulocytes functions typically induced
discussed pathways induced by the chemoattractant agonist (at nanomolar or micromolar doses) and by PMA. The dashed line marked with a star indicates a PKC-dependent Rac activation step. This step is assumed to be deficient in Bt2cAMP-differentiated variant HL60 cells. In these cells, substimulatory doses of the formyl peptide restore a PMA-dependent oxidase response probably through a Rac activation pathway (arrows at left) which is shown to be independent from PI3K and MEK/Erk pathways. See Discussion for commentaries.

by low doses of chemoattractant, i.e., chemotaxis and actin polymerization (42, 46, 68). Although they affected chemotaxis, PI3K inhibitors did not prevent the increase in total F actin in agonist-induced neutrophils (33, 37, 69).

Role of Rac in the complementation of the PMA defect in Bt2cAMP-differentiated variant HL60 cells

Rac regulates the activity of the NADPH oxidase in phagocytic leukocytes by mechanisms that are not fully understood (3, 15). In variant HL60 cells, the restoration of the PMA-dependent respiratory burst following expression of the constitutively active myc-Rac1V12 further underlines a role of Rac in oxidase activation in intact cells. The contribution of Rac as part of the oxidase enzymatic complex has been fairly demonstrated by the requirement of the Rac1/2 protein in oxidant-generating cell-free systems (16, 17, 20) and by the agonist-induced translocation of Rac to the plasma membrane (24, 25). In addition to its role in switching on oxidant formation at the level of the plasma membrane, Rac also appeared to be connected with upstream signaling mechanisms. It has been previously reported that the p21-activated kinase (PAK) and its upstream regulator Rac regulate the activity of p38 SAPK (70–72). PAK also proved to phosphorylate p47(phox) in vitro in a Rac-GTP-dependent manner (73). The involvement of p38 SAPK in the signal pathway leading to superoxide production has been recently underscored in several studies (38, 41, 47–50). Therefore, the question arises as to whether the restoration of the respiratory burst with the active mutant Rac1V12 takes place at the level of the oxidase complex or in upstream signaling. However, in Bt2cAMP-differentiated variant cells, endogenous Rac is supposedly unaltered based upon the fact that the enzymatic complex is functional under chemoattractant stimulation. The weak ability of PMA-challenged Bt2cAMP-differentiated cells to secrete oxidant is much probably due to an inefficient signaling upstream of Rac. Although the phenotype is not expressed in DMSO-differentiated cells, one cannot exclude that the defect does not exist since it could be compensated by the higher level of expression of the NADPH cytosolic factors, p47(phox) and Rac1. Because PMA-induced oxidase could be restored either by addition of low doses of chemoattractant or by expression of myc-Rac1V12, we suggest that Rac belongs to the second aforementioned, PI3K- and MEK-independent pathway. As suggested above, this second pathway may be part of the pathway involved in chemotaxis and F-actin assembly for Rac is also known to regulate cytoskeleton rearrangements (74). Interestingly, Arcaro et al. (75) recently reported that the chemoattractant-dependent activation of Rac in neutrophils triggered the uncapping of actin filaments, independently of PI3K.

In summary, PMA and low concentrations of chemoattractant activate two distinct, restricted pathways that complement each other to fulfill the requirements for NADPH oxidase activation. As illustrated in Fig. 9, at high concentrations of chemoattractant the array of activated effector enzymes overlaps with that activated by PMA alone, leading to NADPH oxidase activation. The observation that a PMA-dependent activation is restored in cells transfected with myc-Rac1V12 cDNA suggests that, in Bt2cAMP-differentiated cells, a cross-talk between a PMA-activated PKC pathway and components upstream the activation of Rac is impaired. The reason of this defect is unknown but may result from a lower expression of regulatory components involved in PMA-mediated Rac activation in cells differentiated with Bt2cAMP. It is tempting to speculate that low concentrations of chemoattractant can restore PMA-mediated NADPH activation by stimulating a WT- and PD98059-resistant signaling pathway in which Rac plays a pivotal role. The possibility to stably transfet the cells and the sensitivity of the superoxide production assay (especially when using low concentrations of chemoattractant combined with PMA) make this variant HL60 cell line an interesting tool to decipher the hierarchy of signaling events upstream and downstream of Rac.

Aknowledgments

We thank Dr M. Dinauer (Indianapolis, IN) for kindly providing the pEF-neo plasmid and Dr P. Chavrier (Marseille, France) for kindly providing the pMT90 plasmid.

References


