Isoquinolinesulfonamide Protein Kinase Inhibitors H7 and H8 Enhance the Effects of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) on Neutrophil Function and Inhibit GM-CSF Receptor Internalization

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Human granulocyte-macrophage colony-stimulating factor (GM-CSF) increases neutrophil surface expression of the cellular adhesion molecule CD11b and primes the respiratory burst stimulated by the bacterial peptide f-met-leu-phe (FMLP). We have examined the effects of the isoquinolinesulfonamide protein kinase inhibitors H7 and H8 on these functions of GM-CSF using whole blood assays. Concentrations of H7 and H8 that inhibited the 12-O-tetradecanoyl-phorbol-13-acetate (TPA) stimulated upregulation of CD11b expression and activation of the respiratory burst, both augmented the effects of GM-CSF. H7 and H8 enhanced the GM-CSF-stimulated increase in CD11b expression to 215% ± 10% (P < .05) and 233% ± 45% (P < .05), respectively, of the value obtained with GM-CSF alone. The GM-CSF priming of the FMLP-stimulated oxidative burst was increased to 190% ± 44% (P < .01) by preincubation with H7 and to 172% ± 25% (P < .01) with H8. Preincubation with H8 did not affect overall binding of 125I-GM-CSF to neutrophils, but inhibited GM-CSF receptor internalization after ligand binding (P < .05). These data indicate that the effects of GM-CSF are not mediated by protein kinase C and that a phosphorylation event down-regulates the neutrophil response to GM-CSF. It suggests that internalization of the receptor-ligand complex is not a rate-limiting step in signal transduction, and that regulation of the rate of internalization may be an important level of control of the activity of GM-CSF.

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KINASE INHIBITORS ENHANCE GM-CSF EFFECTS

MATERIALS AND METHODS

Materials. Purified recombinant human GM-CSF was supplied by Hoechst/Behringwerke (Hounslow, UK/Marburg, W. Germany) and was dissolved in RPMI (GIBCO, Paisley, Scotland), stored at \(-20^\circ\text{C}\) in aliquots of 1 \(\mu\text{g}/\text{mL}\), and thawed on the day of use. TPA (Sigma, Poole, UK) was dissolved in DMSO to a stock concentration of 1 mg/mL and kept at \(-20^\circ\text{C}\) until the day of use. FMLP (Sigma) was dissolved in DMSO to a stock concentration of 5 mg/mL and kept at \(-20^\circ\text{C}\) until the day of use. The protein kinase inhibitor H7 was obtained from Sigma, and H8 and HA1004 from Seikagaku (St Petersburg, FL), constituted as per the manufacturers' instructions to stock 10 mmol/L solutions, and stored at 4°C. 2,7 dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR), dissolved in DMSO to 100 mmol/L concentration, and kept at \(-20^\circ\text{C}\). A CD11b monoclonal antibody (antibody 44) was kindly donated by Dr Nancy Hogg (Imperial Cancer Research Fund, London, UK). Radiolabeled \(^{125}\text{I}\)-GM-CSF, with a specific activity of 60 to 120 \(\mu\text{Ci}/\mu\text{g}\) protein, was obtained from Amersham International (Bucks, UK).

Neutrophil CD11b expression. Whole blood samples were obtained from healthy adult volunteers. Fresh heparinized blood was taken into polystyrene tubes and incubated with TPA (100 ng/mL), GM-CSF (1 ng/mL), or diluent control for 30 minutes at 37°C. Experiments using protein kinase inhibitors included a 10-minute preincubation at 37°C with 1 mmol/L H7, 100 \(\mu\text{mol}/\text{L}\) H8, or 1 mmol/L HA1004. After stimulation with the appropriate agonist, samples were immediately placed on ice and incubated for 30 minutes with saturating doses of CD11b antibody. Initial experiments used UCHT1 as an irrelevant immunoglobulin G1 (IgG1) antibody control; as no significant difference was seen between samples treated in this way and those incubated with diluent (RPMI), subsequent experiments were conducted with diluent control only. After three washes the cells were further incubated with fluorescein-conjugated rabbit anti-mouse antibody (Dako, Bucks, UK) for 30 minutes at 4°C. Samples were then processed using the Coulter Immunoprep Workstation (Coulter, Hialeah, FL), which lyses red blood cells (RBCs) and fixes white blood cells. Analysis was performed by flow cytometry using a Coulter Epics-C machine, the neutrophils being selectively gated by virtue of their light-scattering properties. Assessment of CD11b expression was based on mean cell fluorescence using a linear scale.

Neutrophil \(\text{H}_2\text{O}_2\) production. This was measured using a modification of the method of Bass et al.\(^a\) In brief, freshly drawn whole blood was incubated with 100 \(\mu\text{mol}/\text{L}\) DCF-DA for 15 minutes at 37°C. Experiments examining the effect of protein kinase inhibitors on the respiratory burst included a 10-minute incubation at 37°C with the respective inhibitor or diluent control after this step. This was followed by incubation with 1 ng/mL GM-CSF for 45 minutes at 37°C, followed by the final agonist (TPA/FMLP) for 10 minutes. The reaction was stopped by placing the samples on ice, and they were then prepared for analysis by flow cytometry as previously described. An estimate of total \(\text{H}_2\text{O}_2\) production was made for a given sample by multiplying the percent of responding cells by their mean cell fluorescence.

GM-CSF receptor assay. Purified neutrophils were obtained by first preparing leukocyte-rich plasma by dextran sedimentation of whole blood followed by layering onto a double-density gradient composed of Histopaque 1119 (Sigma) and Lymphoprep 1077 (Nycomed, Oslo, Norway), and centrifuging at 700g for 30 minutes. Neutrophils were harvested from the Lymphoprep/Histopaque interface, washed twice, and resuspended in RPMI with 2% fetal calf serum (FCS). Neutrophils, 5 \times 10^6, were used per point and experiments examining the effect of protein kinase inhibitors included a 10-minute preincubation at 37°C at this stage. Cells were incubated with \(^{125}\text{I}\)-GM-CSF at 37°C, and assessment of nonspecific binding was made by incubation with greater than 100-fold excess cold GM-CSF. At specified time points, total cell-bound activity was measured after centrifugation through 20% glycerol at 7,000g for 60 seconds and freezing the pellet in the liquid phase of liquid nitrogen. Measurement of internalization of receptor bound \(^{125}\text{I}\)-GM-CSF was performed by subjecting an equivalent number of cells to a low pH wash in 3% acetic acid to dissociate membrane-bound \(^{125}\text{I}\)-GM-CSF and counting the cell pellet-associated activity as above. All points were assessed in duplicate.

RESULTS

Effect of GM-CSF and TPA on neutrophil CD11b expression. Both TPA and GM-CSF are known to increase the surface expression of neutrophil CAMs.\(^a\) In initial experiments, a direct comparison of the responses to TPA (100 ng/mL) and GM-CSF (1 ng/mL) was made between purified leukocytes (depleted of RBCs by dextran sedimentation) and whole blood from three normal individuals. Results from these experiments (expressed as percent of control response) gave mean (±SEM) values for the response to TPA of 236 ± 43 and 146 ± 25, and for the response to GM-CSF of 151 ± 10 and 138 ± 40 for whole blood and separated cells, respectively. This indicates that the effects of GM-CSF and TPA on CD11b expression are at least as great in whole blood as in cells that have been partially purified.

In 12 experiments, GM-CSF at a concentration of 1 ng/mL (70 pmol/L) was incubated with whole blood for 30 minutes at 37°C. Neutrophil CD11b expression measured by mean cell fluorescence increased to a mean of 213% ± 28% of control. The data in Fig 1 show that GM-CSF concentrations as low as 250 pg/mL (17 pmol/L) caused an increase in CD11b expression, with a maximal response at 1 ng/mL. The effect of TPA (100 ng/mL) on CD11b expression was also examined. Results from 16 experiments show an increase in CD11b expression to a mean of 274% ± 43% of control. A time course experiment showed that the response to stimulation by GM-CSF in whole blood was rapid, with a measurable increase in CD11b expression by 5 minutes, and near maximal response by 30 minutes (Fig 2). There was only a small increase in CD11b expression with time in the control group incubated with diluent alone, in contrast to a more marked rise reported in experiments using separated cells.\(^b\)

The question of endotoxin contamination leading directly to upregulation of CD11b expression or enhancing the effect of GM-CSF was also addressed. Varying concentrations of endotoxin were added to whole blood with and without GM-CSF, and incubated for 60 minutes at 37°C; no increase in CD11b expression (compared with control samples without endotoxin) was seen in either group at concentrations of endotoxin up to and including 100 ng/mL. The endotoxin contamination of the preparation of GM-CSF used as measured by the limulus amebocyte assay was 0.3 pg/ng.

Effect of GM-CSF on the neutrophil respiratory burst. We have recently shown, using the whole blood fluorimetric technique, that stimulation by 10\(^-6\) mol/L FMLP alone causes only a modest increase in neutrophil \(\text{H}_2\text{O}_2\) production.\(^b\)
Results from 26 separate experiments show a mean response to FMLP stimulation of 559 ± 108 versus 232 ± 36 in unstimulated cells (expressed as product of percent positive cells and their mean cell fluorescence). This response can be markedly enhanced to 3,550 ± 662 by preincubation with 1 ng/mL GM-CSF for 45 minutes at 37°C. The dose-response curve for the GM-CSF priming of the FMLP-induced respiratory burst is similar to that obtained for direct stimulation of CD11b expression, with a maximal effect at 1 ng/mL (70 pmol/L) GM-CSF (Fig 1).

Effect of protein kinase inhibitors on neutrophil responses to TPA. Prior incubation of whole blood with the kinase inhibitors H7 and H8 (10 minutes at 37°C) caused significant inhibition of both TPA (100 ng/mL) induced upregulation of neutrophil CD11b expression (Fig 3A) and the TPA (1 μg/mL) stimulated respiratory burst (Fig 3B). H7, 1 mmol/L, inhibited the TPA-stimulated increase in CD11b expression by a mean of 65% ± 8.8% (n = 8, P < .01 by paired t-test) and TPA-stimulated H₂O₂ production by 94% ± 4.9% (n = 4, P < .05). H8, 100 μmol/L, inhibited CD11b expression by 38% ± 16% (n = 5, P < .01) and H₂O₂ production by 72% ± 14% (n = 4, P < .05). Preincubation with 1 mmol/L HA1004 had no significant effect on either TPA-mediated CD11b expression or respiratory burst (n = 4).

In three experiments examining the effect of H7 and H8 on the TPA-mediated increase in CD11b expression, a lower dose of TPA (10 ng/mL) was used. A more marked inhibition of the upregulation of CD11b was observed with H7 inhibiting the TPA response by 76% ± 16% and H8 by 62% ± 14%.

Effect of protein kinase inhibitors on neutrophil responses to GM-CSF. Prior incubation of whole blood with H7 and H8 caused significant enhancement of both GM-CSF-induced neutrophil CD11b expression (Fig 4A) and the GM-CSF-primed FMLP-stimulated respiratory burst (Fig 4B). Preincubation with H7 gave results of 215 ± 10 (expressed as percent of response seen without H7) for the
The effect of H7 on GM-CSF–stimulated CD11b expression was also examined in partially purified neutrophils prepared by dextran sedimentation of whole blood. Results from these experiments were consistent with findings using whole blood techniques: preincubation with 1 mmol/L H7 inhibited TPA-stimulated CD11b expression completely ($n = 2$) and enhanced the GM-CSF–stimulated increase to $254\% \pm 8\%$ of the response seen without H7 ($n = 3$).

Incubation of blood with FMLP increases neutrophil CD11b expression with a maximal effect at $10^{-10}$ mol/L (data not shown). The response is present at $10^{-11}$ mol/L but is suboptimal (approximately 80% of maximum). To assess whether protein kinase inhibition with H7 was having a nonspecific effect on neutrophil degranulation induced by surface receptor bound agonists, we examined the effect of preincubation with H7 on this response. In contrast to the findings with GM-CSF, 1 mmol/L H7 did not significantly

GM-CSF–stimulated increase in CD11b expression ($n = 6$, $P < .01$) and 190 ± 44 for the GM-CSF–primed FMLP respiratory burst response ($n = 8$, $P < .01$). The enhancement of GM-CSF priming of the respiratory burst by H7 was due to an increase in both the percent of responding cells (from $56 \pm 11$ to $72 \pm 7$) and their mean cell fluorescence (from $54 \pm 15$ to $74 \pm 28$). Typical flow cytometric histograms of the effects of H7 on GM-CSF–stimulated CD11b expression and on the GM-CSF–primed FMLP respiratory burst are shown in Fig 5. Similar results were seen with H8, which enhanced the GM-CSF–mediated increase in CD11b expression to $233 \pm 45$ (n = 4, $P < .05$) and the respiratory burst to $172 \pm 25$ (n = 8, $P < .01$). Neither H7 nor H8 alone had any significant effect on CD11b expression; neither agent caused detectable H$_2$O$_2$ production or had any significant effect on the unprimed FMLP-stimulated respiratory burst. Control experiments ($n = 5$) with 1 mmol/L HA1004 showed no significant effect on GM-CSF–induced CD11b expression or on the GM-CSF primed respiratory burst.
Effect of protein kinase inhibitors on GM-CSF receptor kinetics. The enhancement of the effects of GM-CSF on neutrophil functions by protein kinase inhibition may be mediated by changes in receptor expression, ligand-receptor interaction, receptor internalization, or at later stages in the signal transduction pathway. Therefore, studies on neutrophil GM-CSF receptor expression and internalization were performed using \(^{125}\text{I}\)-GM-CSF.

Preincubation of neutrophils with 100 \(\mu\text{mol/L}\) HA1004 did not reduce receptor internalization.

**DISCUSSION**

In this study we used whole blood assays to examine the effects of GM-CSF on neutrophil function and describe the modulation of these effects by protein kinase inhibitors. We used these techniques because there is increasing evidence from work done in our laboratory and others that neutrophil isolation procedures may themselves alter phagocyte responses. The production of \(\text{H}_2\text{O}_2\), as detected by DCF-DA, is dependent on respiratory burst activity, although the response is augmented by myeloperoxidase (MPO), and there is potential competition for the \(\text{H}_2\text{O}_2\) produced between DCF-DA and cellular reducing agents such as catalase and glutathione. In the whole blood system, FMLP causes detectable \(\text{H}_2\text{O}_2\) production in only a small proportion of neutrophils, but the total response is increased six- to sevenfold by preincubation with GM-CSF. In experiments where we studied purified neutrophils, nearly all respond to FMLP (as assessed by the DCF-DA detection of \(\text{H}_2\text{O}_2\) production and by NBT reduction), and the total respiratory burst activity measured by the superoxide dismutase-inhibitable reduction of ferricytochrome \(c\) is increased approximately threefold by preincubation with GM-CSF. Using the latter assay, the extracellular superoxide measured is not modulated to the same degree by intracellular MPO and reducing systems. This suggests that GM-CSF augments the respiratory burst itself, although the effects of GM-CSF on MPO and glutathione levels have not been determined. The apparently greater effect of GM-CSF in whole blood is probably due to the partial priming of the neutrophils induced by the purification procedures, with an increase in the baseline values. The effects of GM-CSF on the up-regulation of CD11b in whole blood are similarly at least as great as when partially purified leukocytes are used, and this supports the idea that the upregulation of CAMs is a direct effect of GM-CSF. The fact that neutrophil CAM expression does not increase during a 2-hour incubation of whole blood at 37°C without GM-CSF indicates that the process of venesection and incubation per se does not activate the neutrophils. This contrasts with the finding in partially purified neutrophils where in vitro incubation at 37°C causes a progressive rise in CAM expression.

To examine the possible mechanisms of GM-CSF signal transduction in neutrophils, we have looked at the effects of protein kinase inhibitors on GM-CSF stimulation of neutrophil functions in whole blood. The isoquinolinesulfonamides are a group of compounds that inhibit a range of protein kinases with varying degrees of specificity. H7, H8, and HA1004 are all potent inhibitors of CAMP-dependent protein kinase; in addition they inhibit PKC to varying degrees, with H7 and H8 having high activity and HA1004 relatively little. Our results provide indirect evidence of the ability of H7 and H8 to inhibit PKC by significantly reducing the effects of TPA, a direct activator of PKC, on both neutrophil CAM expression and respiratory burst. In keeping with its reported activity, HA1004 had no significant effect on
neutrophil responses to TPA. We show that H7 and H8 do not inhibit either the direct effect of GM-CSF on CAM expression or GM-CSF priming of the FMLP-stimulated respiratory burst, indicating that the effects of this cytokine are not mediated by PKC and confirming the recent results of Mege et al.\textsuperscript{11} Preincubation with HA1004 did not affect neutrophil responses to GM-CSF. We have shown further that preincubation with H7 and H8 causes significant enhancement of GM-CSF–stimulated neutrophil responses and that this augmentation is not seen with HA1004. This suggests that a protein kinase-mediated phosphorylation step is important in the down-regulation of neutrophil responses to GM-CSF. Other studies have reported protein phosphorylations in response to stimulation by this growth factor,\textsuperscript{14,15} and it has been suggested that these may play a role in stimulating further responses relevant to cell survival and proliferation. The data presented here suggest that protein phosphorylation in response to GM-CSF may not only be a stimulatory signal but may play a part in the down-regulation of the cellular response to this growth factor. The lack of specificity of H7 and H8 do not allow conclusions to be made as to what type of kinase activity is down-regulating the GM-CSF response. It appears that GM-CSF does not activate PKC, and the lack of effect of HA1004 suggests that
the effects of GM-CSF are not mediated by cAMP-dependent protein kinases. Further studies will be required to address this issue.

The enhancement of the effects of GM-CSF on neutrophil function by protein kinase inhibition was further investigated by examining the actions of these inhibitors on GM-CSF receptor expression and internalization after ligand binding. The results show that preincubation with H8 or H7 did not alter overall binding of 125I-GM-CSF to its receptors on the cell surface, but that they reduced markedly the rate and degree of receptor internalization following 125I-GM-CSF binding. In conjunction with our observation that these protein kinase inhibitors enhance the functional effects of GM-CSF, we can conclude that internalization of receptor-bound GM-CSF is not a rate-limiting step for the action of this cytokine. Receptor-mediated endocytosis is a mechanism by which many different cell types internalize macromolecules, including several growth factors.24 The biologic role of receptor-mediated internalization is varied and system-specific: it appears to be a prerequisite for the action of the cytokine interleukin-1 (IL-1), which after internalization is transported to the cell nucleus,25 and variant cells unable to internalize IL-1 are unresponsive to stimulation.26 In contrast, the binding of several growth factors, including epidermal growth factor (EGF)27 and M-CSF,28 is followed by internalization and intracellular degradation and acts as a mechanism by which their effects are down-regulated.

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