Interactions of Granulocyte-Macrophage Colony-Stimulating Factor (CSF), Granulocyte CSF, and Tumor Necrosis Factor α in the Priming of the Neutrophil Respiratory Burst

By Asim Khwaja, Julia E. Carver, and David C. Linch

Exposure of neutrophils to a range of cytokines augments their response to subsequent agonist-induced activation of the respiratory burst. We have examined the effects of several of these factors, both singly and in combination, on the priming of f-met-leu-phe (FMLP) and complement C5a-stimulated neutrophil H2O2 production, using a whole blood flow cytometric assay designed to minimize artefactual activation. Both granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor α (TNFα) produced a similar degree of priming of the FMLP-stimulated burst in vitro (568% ± 86%, n = 41, and 581% ± 95%, n = 21, of the response seen with FMLP alone, respectively), but with markedly different kinetics (half-maximal response 20 minutes and 7 minutes, respectively). Preincubation with granulocyte colony-stimulating factor (G-CSF) alone caused only modest priming (202% ± 39%, n = 14). Priming with cytokine combinations of the FMLP-stimulated burst showed that the combinations of G-CSF and TNFα and GM-CSF and TNFα are highly synergistic, with recruitment of neutrophils unresponsive to priming by single agents. Priming with the combination of GM-CSF and G-CSF was not significantly different to priming with GM-CSF alone. Similar results were obtained using C5a as the respiratory burst stimulus. Significant priming of the FMLP-stimulated respiratory burst was seen in vivo in patients receiving an infusion of GM-CSF (332% ± 50% of preinfusion response to FMLP, P < .005, n = 8). Priming was also seen in patients receiving G-CSF (152% ± 58%, n = 5), although this did not reach conventional significance levels (0.05 < P < .1). Although GM-CSF infusion caused priming in vivo, this was 48% less than predicted by preinfusion in vitro responses. This result was not due to inadequate GM-CSF levels as addition of further GM-CSF ex vivo did not correct the response. However, these neutrophils were still able to respond appropriately to ex vivo priming with TNFα, with a doubling in H2O2 production.

THE PHAGOCYTE respiratory burst plays a central role in normal host defence.1 The formation of oxidative burst products is stimulated by bacterial phagocytosis, and by agonists such as formyl-methionyl bacterial peptides (FMLP), complement-derived C5a, and phorbol ester. In neutrophils, this process may be enhanced, or ‘primed,’ by prior short-term exposure to molecules such as the granulocyte-macrophage colony-stimulating factor (GM-CSF)2 and granulocyte colony-stimulating factor (G-CSF),3 and other cytokines including tumor necrosis factor α (TNFα) and interleukin-1 (IL-1).4 The presence of these cytokines at sites of infection may therefore make a significant contribution to neutrophil microbicidal activity.

Many of the studies examining the effects of cytokines on neutrophil priming have been performed on cells subjected to separation procedures that may in themselves alter cellular responses, perhaps by causing partial activation.5 In this study we compare the effects of three cytokines, GM-CSF, G-CSF, and TNFα, on neutrophil priming using a whole blood assay devised to minimize such artefactual activation6 and show that the kinetics and degree of response to priming varies markedly between these cytokines when used alone. In addition, as it is likely that an infective or inflammatory focus will contain a complex mixture of cytokines that may modify neutrophil responses in a manner not predictable from the study of individual cytokines, we have examined the effects of combinations of these molecules on neutrophil priming.

There has been considerable interest in the clinical use of CSFs to not only increase neutrophil numbers, but to also enhance their functional activity.6 We and others have reported that the administration of GM-CSF will prime the neutrophil respiratory burst in vivo,7,8 and similar findings have been reported for G-CSF.9 Although we have seen significant priming of the FMLP-stimulated respiratory burst with GM-CSF infusion, this effect is considerably reduced when compared with preinfusion responses to GM-CSF priming in vitro, and cannot be corrected by further ex vivo incubation with optimal concentrations of GM-CSF.7 This finding raises the possibility that the priming of neutrophils by exogenous cytokines, administered to enhance host defence, may result in the presence of circulating phagocytes unable to respond appropriately to other endogenously produced cytokines at sites of infection. In this study, we further delineate the effects of the systemic administration of GM-CSF on the neutrophil respiratory burst and show that cells exposed to GM-CSF in vivo are still able to respond appropriately to priming with TNFα.

MATERIALS AND METHODS

Patients. Samples were obtained from patients with relapsed Hodgkin’s disease and high grade non-Hodgkin’s lymphomas receiving high-dose chemotherapy, with or without subsequent autologous bone marrow rescue, who were taking part in trials to assess the effects of growth factors on hematopoietic reconstitution. Samples were taken before and after a 2-hour test intravenous infusion of GM-CSF at 15 μg/m2/h before the start of chemotherapy (three patients), or after the completion of chemotherapy but before autologous bone marrow rescue (three patients), or after the completion of chemotherapy but before autologous bone marrow rescue (three patients).

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before chemotherapy-induced leukopenia had occurred (five patients). Responses observed before starting chemotherapy were comparable with those seen after the completion of chemotherapy. Samples from patients receiving G-CSF were taken before and at 60 minutes after the completion of a 30-minute intravenous infusion at doses ranging from 5 to 20 μg/kg after the completion of chemotherapy but before chemotherapy-induced leukopenia had occurred. Growth factors were administered into a central vein by means of an indwelling Hickman catheter (C.R. Bard Inc, Cranston, RI). Local ethical committee approval and full informed written consent from each patient were obtained.

Cytokines. Recombinant human (rh) GM-CSF expressed in Escherichia coli was provided by Hoechst UK/Behringerwerke (Marburg, Germany) and rhG-CSF expressed in Chinese hamster ovary (CHO) cells was provided by Chugai Pharmaceutical Co (London, UK). rhTNFα was provided by The Immunex Corporation (Seattle, WA).

Materials. All cytokines were dissolved in RPMI with 2% fetal bovine serum (FBS; both from Gibco, Paisley, Scotland), and stored at −70°C until the day of use. FMLP (Sigma, Poole, UK) was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. 2',7'dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR) and dissolved in DMSO to 100 μmol/L concentration and kept at −20°C. Complement C5a was obtained from Sigma and reconstituted with RPMI plus 0.5% bovine serum albumin (Sigma). 125I-GM-CSF with specific activity between 800 and 1,200 Ci/mmol was obtained from Amersham (Bucks, UK).

Respiratory burst assay. Neutrophil H2O2 production was measured using a modification of the method of Bass et al11 as previously described. Blood samples from either normal laboratory personnel or patients were taken into preservative-free heparin and incubated with 100 μmol/L DCF-DA for 15 minutes at 37°C. Subsequent incubation with cytokine or diluent control for 45 minutes at 37°C, or for varying periods during time course experiments, was followed by stimulation with FMLP, or by incubation with diluent control for 10 minutes at 37°C. In experiments in which the effects of cytokine combinations were being examined, these were added simultaneously, unless stated otherwise. In experiments looking at the effects of C5a on the respiratory burst, blood samples were first centrifuged at 800 rpm and autologous plasma removed and then washed twice with RPMI/5% FBS and restored to their original volume with RPMI/5% FBS.

This removal of autologous plasma was necessary as no effect of C5a on neutrophil H2O2 production was observed in whole blood. After stimulation, samples were then taken onto ice, and processed using the Coulter Immunoprep Workstation (Coulter, Hialeah, FL), which lysed red blood cells and fixed 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. The percentage of responding cells was assessed by setting the diluent control sample to approximately 5% positive. An estimate of total H2O2 production was made for a given sample by multiplying the percentage of responding cells by their mean cell fluorescence, as measured on a linear scale, and is described in arbitrary units.

125I-GM-CSF binding assay. Heparinized blood was taken before and at various time points after the end of the GM-CSF infusion. Neutrophils were prepared by dextran sedimentation of red blood cells and buoyant density centrifugation. Neutrophils (106) were incubated with 125I-GM-CSF, with and without a hundred-fold excess of unlabeled GM-CSF to control for nonspecific binding, at 37°C for 45 minutes. All points were run in duplicate. After the binding step, the neutrophils were layered onto a cushion of chilled fetal calf serum, centrifuged for 30 seconds at 8,000g, snap frozen in liquid nitrogen, and the cell pellets sliced and counted in a gamma counter.

Statistical methods. The results of priming with cytokines in vitro and in vivo were compared by paired Student’s t-test and were performed using the Statview package on an Apple Macintosh computer (Cupertino, CA).

RESULTS

Comparative effects of cytokines on neutrophil priming. The effects of preincubation of whole blood with solitary cytokines, at increasing concentrations, on the FMLP-stimulated neutrophil respiratory burst are shown in Fig 1. None of the cytokines tested showed any significant direct activation of the respiratory burst with values (as mean ± SEM percentage of response with diluent control) of 122% ± 19%, 90% ± 30%, and 103% ± 26% for GM-CSF, G-CSF, and TNFα, respectively. GM-CSF priming of the FMLP (10-6 mol/L)-stimulated burst is detectable at 50 pg/mL (3.5 pmol/L) and maximal at 1 ng/mL (70

Fig 1. Dose-response curves of priming of the FMLP-stimulated neutrophil respiratory burst with individual cytokines. Whole blood loaded with DCF-DA was incubated with TNFα (n = 9), GM-CSF (n = 6), or G-CSF (n = 5) at varying concentrations for 45 minutes at 37°C and then stimulated by FMLP (10-6 mol/L) for 10 minutes. Samples were analyzed by flow cytometry with an index of total H2O2 production derived by the product of the percentage of responding cells and their mean cell fluorescence. Results (mean ± SE) are expressed as a percentage of the response seen with FMLP alone (100%). (The baseline FMLP response was 422 ± 123 U for this set of experiments.)
pmol/L). With increasing concentration over this range, there is recruitment of cells into the responding population with an increase in their mean cell fluorescence. G-CSF has relatively modest priming capability in the whole blood assay, and the small increase in total neutrophil \( \text{H}_2\text{O}_2 \) production seen at the optimal concentration of 10 ng/mL (500 pmol/L) is due to the recruitment of small numbers of cells into the responding population, which also has a relatively low level of respiratory burst activity. Priming by TNF\( \alpha \) of total neutrophil \( \text{H}_2\text{O}_2 \) production is seen at concentrations starting at 5 U/mL (10 pmol/L) and is maximal at 500 U/mL (1 nmol/L). The increase in respiratory burst activity seen at the lower end of the concentration range is largely due to recruitment of responding neutrophils (15% ± 7% to 43% ± 13% responding at 5 and 50 U/mL, respectively), and at higher concentrations due to an increase in both numbers of active cells and their mean cell fluorescence. The overall results at optimal concentrations of cytokine for FMLP-stimulated total neutrophil \( \text{H}_2\text{O}_2 \) production (as a percentage of the response seen with FMLP alone) for GM-CSF (1 ng/mL) was 558% ± 86% (n = 41), for TNF\( \alpha \) (100 U/mL) 581% ± 95% (n = 21), and for G-CSF 202% ± 39% (n = 14). A similar pattern of results was seen using complement C5a as the respiratory burst stimulus with priming by GM-CSF to 474% ± 153% (n = 6) with the response with C5a alone, by TNF\( \alpha \) to 429% ± 130% (n = 7), and G-CSF to 119% ± 37% (n = 6).

**Time course of priming with single cytokines.** The respective time courses of priming with single cytokines on the FMLP-stimulated respiratory burst are shown in Fig 2. Priming with GM-CSF or G-CSF requires at least 10 to 15 minutes preincubation before significant enhancement of \( \text{H}_2\text{O}_2 \) production is seen, with maximal effects by 30 minutes. In contrast, priming with TNF\( \alpha \) is rapid, with detectable activity within 2 minutes and maximal effects by 15 minutes.

The comparative effects of GM-CSF and G-CSF on in vivo priming of the respiratory burst. Neutrophil respiratory burst activity was measured both before and after the intravenous infusion of rhGM-CSF at a dose of 15 \( \mu \)g/m\(^2\)/h over 2 hours in patients with relapsed lymphomas. Examination of in vitro responses before the infusion showed results falling within the range for healthy controls (Table 1). Priming of the FMLP response was seen in vivo, with an increase from 275% ± 59% to 912% ± 138% (expressed as the percentage of response obtained with no stimulus in vitro, \( P < .005 \) by paired \( t \)-test, \( n = 8 \)). However, when compared with the in vitro preinfusion mean response to priming with GM-CSF of 1,735% ± 310%, this was significantly reduced (\( P < .01 \)). This finding extends our data as previously reported. The question of whether exposure to initial suboptimal concentrations of GM-CSF in vivo leads to the downregulation of subsequent neutrophil responses to this cytokine was addressed by reproducing the infusion conditions in vitro. Results from two separate experiments in which samples of blood were either exposed to gradually increasing concentrations of GM-CSF (from 10 pg/mL to 1 ng/mL) or to optimal concentrations from the outset did not show any difference in the degree of respiratory burst priming. Furthermore, the addition of an optimal concentration of GM-CSF (10 ng/mL) ex vivo to neutrophils taken from patients after a GM-CSF infusion did not lead to any further increase in \( \text{H}_2\text{O}_2 \) production (836% ± 156%), suggesting that the reduced response was not attributable to exposure to suboptimal levels of GM-CSF in vivo. A time course of \( \text{H}_2\text{O}_2 \) production was performed in one patient after GM-CSF infusion. This time course showed that the in vivo priming effect of GM-CSF had virtually disappeared 2 hours after the end of the infusion and that this coincided with the restoration of ex vivo priming with added GM-CSF to preinfusion levels. In a single experiment, blood was taken at various time points before and after the end of GM-CSF infusion and an assessment of neutrophil binding of \( ^{125} \text{I} \)-GM-CSF (per 10\(^6\) cells) was made after the removal of exogenous unlabeled free GM-CSF. This experiment showed that \( ^{125} \text{I} \)-GM-CSF binding decreased to less than 5% of the preinfusion level after the completion of the
Table 1. Comparison of In Vitro and In Vivo Priming of the FMLP-Stimulated Neutrophil Respiratory Burst by GM-CSF (n = 8)

<table>
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<th>In Vitro GM-CSF (preinfusion)</th>
<th>In Vivo GM-CSF (postinfusion)</th>
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<tr>
<td>Nil</td>
<td>100</td>
<td>130 ± 32</td>
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<tr>
<td>FMLP</td>
<td>275 ± 59</td>
<td>1,735 ± 310*</td>
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<tr>
<td>TNFa + FMLP</td>
<td>1,070 ± 232</td>
<td>2,418 ± 225‡</td>
<td>1,591 ± 2429†</td>
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</tbody>
</table>

Patient samples taken before GM-CSF infusion (15 μg/mL for 2 hours) were stimulated directly with FMLP (10-4 mol/L), or first incubated with GM-CSF (1 ng/mL), TNFa (100 U/mL), or a combination of the two. Samples taken after GM-CSF infusion were stimulated with FMLP both before and after further TNFa incubation in vitro (n = 5). Results are given as a percentage of preinfusion control response.

*Significant enhancement of preinfusion response to FMLP alone (P < .001).
†Significant enhancement of preinfusion response to FMLP alone (P < .005).
‡Significant enhancement of effect of GM-CSF alone (P < .025) or TNFa alone (P < .025).
§Significant enhancement of response to in vivo GM-CSF priming (P < .01).

infusion, indicating a lack of available free neutrophil surface GM-CSF receptors.

The effect of the administration of intravenous rhG-CSF on neutrophil priming was examined in five patients receiving doses ranging from 5 to 20 μg/kg. In contrast to the greater than threefold priming seen with GM-CSF, only relatively modest priming was seen after G-CSF infusion with an increase in the response to stimulation with FMLP from 421% ± 242% to 641% ± 246% (.05 < P < .1, n = 5). This was not significantly affected by subsequent ex vivo incubation with an optimal G-CSF concentration (677% ± 215%) or significantly different from the preinfusion in vitro priming with G-CSF (739% ± 280%).

The effect of cytokine combinations on the in vitro neutrophil respiratory burst in healthy controls. The effect of preincubation of whole blood with individual cytokines and combinations of cytokines on the neutrophil respiratory burst was examined in six healthy volunteers and the data are shown in Table 2. Priming with GM-CSF of the FMLP-stimulated burst in this group of individuals led to an increase in total H2O2 production from a mean (±SE) of 755 ± 147 fluorescence units for FMLP alone to 4,025 ± 1,101 (P < .01 by paired t-test). Priming with G-CSF alone leads to only modest overall enhancement of respiratory burst activity (1,227 ± 370). Preincubation of whole blood in the same individuals with TNFa resulted in a marked increase of total H2O2 production to 4,896 ± 1,467 (P < .01). This finding is in accord with the data obtained from the larger number of individuals described above.

The response to priming with the combination of G-CSF and GM-CSF was not significantly different than the response seen with GM-CSF alone and this was confirmed in mixing experiments over a wide concentration range of both cytokines. In contrast, the combination of priming with G-CSF (50 ng/mL) and TNFa (100 U/mL) before stimulation with FMLP gave significantly greater total H2O2 production when compared with TNFa alone (22% increase, P < .05). Representative flow cytometric histograms are shown in Fig 3.

However, because simple mixing experiments at single concentrations of cytokines can give an erroneous impression of their interaction, isoboles were constructed to reliably determine these effects. These are lines of equal effect (usually a fixed proportion of maximum effect, eg, 50% maximum) derived from multiple dose response curves of agent 1 in the presence of various concentrations of agent 2. The concentrations of the two agents that singly and in various combinations induce the selected effect are plotted against each other. Upwardly concave plots indicate that less cytokine is required in combination than when either agent is used separately and therefore indicate a synergistic interaction. Additive interactions show a linear plot, and antagonistic interactions show an upwardly convex curve. Checkerboard analysis across a range of G-CSF and TNFa concentrations showed that this was a highly synergistic combination with regard to priming of the FMLP-stimulated burst (Fig 4A).

The combination of GM-CSF (1 ng/mL) with TNFa (100 U/mL) also gave significantly greater total H2O2 production when compared with TNFa alone (57% increase, P < .025). Isobole construction shows that the combination of these two cytokines is also highly synergistic (Fig 5A). The increased response seen with the combination of GM-CSF and TNFa, when compared with either cytokine alone, was attributable to an increase in both the percentage of responding cells (61% v 75%, at optimal concentrations) and in their mean cell fluorescence (72 v 97).

A second agonist that stimulates the respiratory burst, C5a (10 ng/mL), was also used to stimulate cells after cytokine priming. Figures 4B and 5B show that the synergistic interactions of G-CSF with TNFa and of GM-CSF with TNFa are also seen using this agonist. The lack of synergism between GM-CSF and G-CSF seen using FMLP was confirmed with C5a stimulation.

Careful time courses of the effects of cytokines in combination (at optimal concentrations as above) were performed in three separate experiments and the results are shown in Fig 6. Figure 6 shows that the synergism between GM-CSF and TNFa is seen between 15 and 30 minutes after the addition of these cytokines and also confirms the absence of synergy between GM-CSF and G-CSF at any point during the priming process. Due to the modest enhancement of the TNFa response by the addition of G-CSF, it is difficult to comment definitively as to the timing of the synergy seen between these two molecules. Further characterization of the interactions between these cytokines was performed by varying their sequence of addition. The preaddition of TNFa (30 minutes) followed by GM-CSF (45 minutes) led to a modest reduction in response (653% ± 272% of the response to FMLP alone, mean ± SEM) when compared with the reverse situation of GM-CSF preceding TNFa (782% ± 308%, n = 8, P < .05 by paired t-test). A similar trend was seen when TNFa addition preceded that of G-CSF (453% ± 203%) when compared with the reverse situation of G-CSF followed by TNFa (593% ± 268%), although this difference failed to
CMOKINE INTERACTIONS IN NEUTROPHIL PRIMING

Table 2. The Effect of Single and Combinations of Cytokines on the Priming of the FMLP \(10^{-4}\) mol/L)-Stimulated Neutrophil Respiratory Burst in Six Normal Individuals

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<td>21</td>
</tr>
<tr>
<td>Product</td>
<td>1,740</td>
<td>—</td>
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The control percentage of positive responding neutrophils is set at 5%. The product of the percentage of positive and the MCF provides an index of the total neutrophil H₂O₂ production per sample. Concentrations of cytokines were GM-CSF 1 ng/mL, G-CSF 50 ng/mL, and TNFα 100 U/mL.

reach conventional levels of statistical significance (.05 < P < .1, n = 6). These reduced responses to cytokine combinations seen with TNFα preincubation were, however, still significantly greater than the responses seen with TNFα alone (380% ± 163%), showing that the neutrophils could still be further primed by both G-CSF and GM-CSF.

The effect of cytokine combinations on the respiratory burst in neutrophils primed in vivo. To assess whether the decreased GM-CSF priming seen in vivo reflected a global reduction in the neutrophil's ability to respond to priming agents after a GM-CSF infusion, samples taken at the end of the infusion were incubated with TNFα and G-CSF and then stimulated with FMLP. No significant increase in H₂O₂ production was seen after incubation with G-CSF, consistent with in vitro findings, but the addition of TNFα resulted in a significant increase in total FMLP-stimulated respiratory burst activity from 827% ± 208% to 1,591% ± 412% (n = 5, P < .01). The increased respiratory burst activity was attributable to an increase in both the percentage of responding cells (from 30% ± 7% to 44% ± 9%) and their mean cell fluorescence (MCF). Although the absolute values of response were reduced, this doubling in respiratory burst activity with the addition of in vitro TNFα to in vivo GM-CSF–primed cells was comparable with the increase seen with the preincubation in vitro combination of these cytokines (2.1-fold).

Neutrophils taken after G-CSF infusion were also assessed for their ability to respond to subsequent incubation with TNFα in vitro. Data from three separate infusions showed that the addition of TNFα in vitro to in vivo G-CSF–primed cells led to a significant increase in FMLP-stimulated H₂O₂ production from a mean of 254% ± 23% to 1,013% ± 242%. Similar to the results seen with in vivo GM-CSF priming, this response was less in absolute terms from that obtained with the combination of G-CSF and
Fig 3. Representative flow cytometric histograms showing the effects of priming of the FMLP-stimulated neutrophil respiratory burst with individual and combinations of cytokines. Whole blood loaded with DCF-DA was incubated with TNFα (100 U/mL), GM-CSF (1 ng/mL), or G-CSF (50 ng/mL), or combinations thereof, for 45 minutes at 37°C and then stimulated by FMLP (10^-8 mol/L) for 10 minutes. Samples were analyzed by flow cytometry with an index of total H₂O₂ production derived by the product of the percentage of responding cells and their mean cell fluorescence. (A) Negative control; (B) FMLP alone; (C) G-CSF + FMLP; (D) GM-CSF + FMLP; (E) TNFα + FMLP; (F) G-CSF with GM-CSF + FMLP; (G) G-CSF with TNFα + FMLP; (H) GM-CSF with TNFα + FMLP.

TNFα in vitro before the infusion (1,527% ± 285%), but was comparable in relative terms (fourfold increase in vivo compared with fivefold increase in vitro seen with the addition of TNFα to G-CSF-primed cells).

DISCUSSION

In this study, we have characterized the effects of three cytokines, GM-CSF, G-CSF, and TNFα, either singly or in combination, on the priming of the FMLP-stimulated neutrophil respiratory burst in whole blood. We have previously shown that, in whole blood, stimulation with FMLP alone causes only modest activation of the respiratory burst. The in vitro responses in blood from normal individuals and patients with malignant lymphomas show that there are significant differences in the ability of the three agents tested when used alone to prime intracellular H₂O₂ production. Preincubation with an optimal concentration of GM-CSF in vitro leads to an approximately fivefold increase in respiratory burst activity when compared with the response to FMLP alone. This priming effect is greater than that generally reported by other investigators using a superoxide anion release assay with isolated neutrophils, in which approximately threefold increases in respiratory burst activity were seen. This result may be due to partial activation consequent upon neutrophil purification procedures leading to a reduced ability to respond to further stimuli, or a reflection of the different assays used. Using the whole blood assay, we have found that preincubation with G-CSF causes only modest priming (twofold), a result that is in keeping with some studies, although others have reported more marked priming (approximately fourfold).

There have been varying reports of the effect of TNFα on the neutrophil respiratory burst. Larrick et al. and Tsujimoto et al. reported direct activation of the burst by TNFα, whereas Atkinson et al. did not detect direct activation, but did find priming of the FMLP-stimulated respiratory burst. More recently, Schleiffenbaum and Fehr have shown that TNFα does not directly stimulate the respiratory burst of neutrophils in suspension, but will cause activation of adherent cells. In this study, we were unable to detect any direct effect of TNFα on activating the respiratory burst in whole blood. However, we have shown that TNFα has potent priming activity, with an increase in FMLP response by approximately sixfold. Time courses of priming using these cytokines showed that, although the final degree of priming seen with GM-CSF and TNFα is similar, the kinetics of the individual responses are markedly different: there is a detectable TNFα effect by 2 minutes, with a near-maximal response by 15 minutes, whereas GM-CSF priming is not detectable until 5 to 10 minutes preincubation, with a near-maximal response by 30 minutes. The kinetics of G-CSF priming are similar to those of GM-CSF.

The effects of combinations of cytokines on priming were also examined in normal individuals and show that subpopulations of neutrophils unresponsive to priming with single
Fig 6. Time course of the effect of single and combination cytokines on priming of the FMLP-stimulated neutrophil respiratory burst. Whole blood loaded with DCF-DA was incubated with (0) TNFα (100 U/mL), (Φ) GM-CSF (1 ng/mL), or (□) G-CSF (50 ng/mL), or combinations thereof, for the indicated times at 37°C and then stimulated by FMLP (10^-8 mol/L) for 10 minutes. Samples were analyzed by flow cytometry with an index of total H₂O₂ production derived by the product of the percentage of responding cells and their mean cell fluorescence (arbitrary units). Results shown are the mean ± SEM of three separate experiments (all standard errors were within 25% of the mean and have been omitted to maintain clarity). (◇) G-CSF and GM-CSF; (■) G-CSF and TNFα; (□) GM-CSF and TNFα; (△) control.

Fig 5. Isobole of the effect of GM-CSF and TNFα priming of the (A) FMLP- and (B) C5a-stimulated neutrophil respiratory burst. Multiple dose-response curves of one cytokine were performed in the presence of various concentrations of the other. The concentrations of the two cytokines that singly or in combination produce a half-maximum effect are plotted against each other. The concave upward plot indicates a highly synergistic interaction between these factors.

Fig 6. Time course of the effect of single and combination cytokines on priming of the FMLP-stimulated neutrophil respiratory burst. Whole blood loaded with DCF-DA was incubated with (0) TNFα (100 U/mL), (Φ) GM-CSF (1 ng/mL), or (□) G-CSF (50 ng/mL), or combinations thereof, for the indicated times at 37°C and then stimulated by FMLP (10^-8 mol/L) for 10 minutes. Samples were analyzed by flow cytometry with an index of total H₂O₂ production derived by the product of the percentage of responding cells and their mean cell fluorescence (arbitrary units). Results shown are the mean ± SEM of three separate experiments (all standard errors were within 25% of the mean and have been omitted to maintain clarity). (◇) G-CSF and GM-CSF; (■) G-CSF and TNFα; (□) GM-CSF and TNFα; (△) control.

Factors may be recruited by combinations of cytokines. In addition, certain combinations of priming agents resulted in an increase in the mean H₂O₂ production of cells responding to preincubation with single cytokines (Table 2). No significant additive effect was seen when the combination of G-CSF and GM-CSF was compared with the response to GM-CSF alone, suggesting that these factors may act on the same population of cells, or via a common postreceptor mechanism. Avalos et al have also recently shown that GM-CSF will partially inhibit G-CSF binding to its receptor on neutrophils, and this may further explain the absence of any additive effect. In contrast, the addition of G-CSF to TNFα resulted in a modest, but significant increase in overall H₂O₂ production when compared with the priming seen with TNFα alone (mean 23% increase, P < .05 by paired t-test), and combination experiments over a wide range of cytokine concentrations showed that this effect was highly synergistic. The combination of TNFα with GM-CSF resulted in a marked increase in H₂O₂ production when compared with either TNFα alone (mean 57% increase, P < .025) or GM-CSF alone (mean 91% increase, P < .025). This result was attributable to increases in both the numbers of responding cells and in their mean cell fluorescence, and was also found to be highly synergistic across a range of cytokine concentrations. Comparable results of synergy between G-CSF and TNFα and GM-CSF and TNFα were shown using complement C5a as the respiratory burst stimulus. There has been a recent report that incubation with TNFα downregulates the expression of the neutrophil GM-CSF receptor, and it has been suggested that this may be relevant in preventing excessive neutrophil activation at sites of inflammation. Similar findings have been reported for the neutrophil G-CSF receptor. We have shown that the preincubation of neutrophils with TNFα followed by the addition of either G-CSF or GM-CSF will lead to small reductions in the overall respiratory burst when compared with the reverse situation in which CSF incubation precedes TNFα addition. How-
ever, this effect is small and does not affect the overall synergy between these factors. The synergistic interactions between both GM-CSF and TNFα, and G-CSF and TNFα, may thus play an important role in producing an effective response to microbial infection, even at relatively low levels of cytokine concentration.

We have also looked at the responses of patients with relapsed lymphoma to priming with these cytokines in vitro and were unable to detect any significant difference between them and normal controls. We and others have previously reported that the clinical administration of GM-CSF leads to priming of the neutrophil respiratory burst in vivo. These results are confirmed here. Although there is marked and significant priming of the oxidative burst in vivo, we have observed that this is significantly reduced when compared with preinfusion values obtained with in vitro incubation with GM-CSF (mean of 48% reduction, P < .025, n = 8), and cannot be corrected by the addition of optimal concentrations of GM-CSF ex vivo. A single binding experiment with 125I-GM-CSF showed that there was a marked reduction in the number of available neutrophil GM-CSF receptors after the infusion. There are several possible reasons that may explain the phenomenon of reduced maximal neutrophil responses seen after GM-CSF infusion. The administration of GM-CSF leads to a rapid decrease in the number of circulating neutrophils, due to margination in the pulmonary vasculature, followed by a relatively early increase above baseline. This increase has been attributed to generalized demargination and possibly to early release from the marrow. The presence of significant numbers of immature neutrophils could potentially cause an apparent reduction in responsiveness. The timing of the sampling in this study, which precedes the increase in neutrophil count, and our previous observation that at this time point after GM-CSF administration there is no significant increase in numbers of immature myeloid cells weighs against this possibility. Another possible explanation for the reduced responsiveness may be that the process of margination/demargination, in some unexplained way, leads to partial neutrophil activation and consequent reduction in responsiveness. Nathan has reported that the addition of either GM-CSF or G-CSF to adherent neutrophils in vitro triggers significant H₂O₂ production. It may be that adherence to pulmonary endothelium during GM-CSF administration causes significant neutrophil H₂O₂ release, and that this leads to a reduction in neutrophil responses to further stimulation ex vivo.

We have further addressed the question of whether this reduction in maximal response represents a generalized blunting of neutrophil responses by the addition of TNFα to in vivo GM-CSF-primed neutrophils, and have shown that these cells are still able to respond appropriately to further priming with this cytokine, with a doubling in respiratory burst activity. This result is similar to the effect seen with the combination of GM-CSF and TNFα in vitro before the infusion of GM-CSF. Only modest priming of FMLP-stimulated neutrophil H₂O₂ production was seen after the infusion of G-CSF in five patients. All G-CSF patients were investigated after the completion of chemotherapy and although this did not have any detectable effect on the neutrophil respiratory burst in pilot experiments (data not shown), the G-CSF group is not strictly comparable with the GM-CSF patients, three of eight of whom were investigated before the start of cytotoxic chemotherapy. However, the data for in vivo G-CSF priming is in keeping with the modest effects seen with this factor in vitro, both in patients and normal volunteers. The lack of marked in vivo priming with G-CSF might contribute to the low toxicity of this factor. Neutrophils from patients receiving G-CSF were able to be primed still further with TNFα in vitro. The fact that G-CSF is synergistic with TNFα suggests that the systemic administration of G-CSF may augment neutrophil responses to lower levels of TNFα produced locally at sites of inflammation.

In this study, we have delineated the effects of single and combination cytokines on the priming of the neutrophil respiratory burst in whole blood, and show that these may have synergistic effects with recruitment of previously unresponsive neutrophils and cause an increase in the activity of responding cells. It has been suggested that the administration of cytokines to enhance neutrophil activity against invading micro-organisms may be of clinical benefit. We have shown that G-CSF has very modest neutrophil-priming activity in whole blood, both in vitro and in vivo, but that it can synergise with TNFα. In contrast, GM-CSF administration causes significant priming of the respiratory burst, although the effect is not as marked when compared with preinfusion values obtained in vitro. Neutrophils taken after GM-CSF infusion are still able to respond appropriately to priming with TNFα and there is marked synergy between these factors in vitro. This finding suggests that, in the presence of systemic GM-CSF, neutrophils may respond to lower tissue concentrations of TNFα. Although G-CSF and GM-CSF cause different degrees of priming of the respiratory burst in vivo, we have not examined the relationship between the degree of priming seen and other tests of neutrophil function, such as microbial phagocytosis and killing. The findings detailed in this study illustrate the complexity of cytokine effects on neutrophil priming in vitro and in vivo and may be relevant to the clinical exploitation of these factors.

REFERENCES


Interactions of granulocyte-macrophage colony-stimulating factor (CSF), granulocyte CSF, and tumor necrosis factor alpha in the priming of the neutrophil respiratory burst

A Khwaja, JE Carver and DC Linch