

Human GM-CSF Primes Neutrophils for Enhanced Oxidative Metabolism in Response to the Major Physiological Chemoattractants

By Richard H. Weisbart, Luz Kwan, David W. Golde, and Judith C. Gasson

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a T cell-derived lymphokine which induces hematopoietic precursor cells to proliferate in vitro and differentiate to neutrophils and macrophages. GM-CSF also inhibits the motility of mature neutrophils (NIF-T activity), and primes neutrophils to enhance oxidative metabolism in response to the bacterial chemoattractant, *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP). The present study was designed to determine whether this lymphokine also enhances neutrophil oxidative metabolism in response to the other major physiological chemoattractants which include complement-derived C5a, and the 5-lipoxygenation product of arachidonic acid, leukotriene B₄ (LTB₄). Su-

PRODUCTS OF OXIDATIVE metabolism mediate neutrophil (PMN) inflammatory responses and play an important role in host defense.¹ Bacterial-derived *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP), complement-derived C5a, and the 5-lipoxygenation product of arachidonic acid metabolism, leukotriene B₄ (LTB₄) are chemotactic factors for PMN that initiate PMN inflammatory responses by activating oxidative metabolism.²⁻⁵ Recent studies identified a T lymphocyte-derived granulocyte-macrophage colony-stimulating factor (GM-CSF) that primes PMN for enhanced oxidative metabolism in response to f-MLP.⁶⁻⁸ Superoxide anion production was increased three- to fourfold in response to f-MLP by PMN preincubated with purified biosynthetic GM-CSF. Purified GM-CSF alone did not stimulate superoxide anion production. Since C5a and LTB₄ are also chemotactic, it is of considerable interest to determine whether GM-CSF also primes PMN to enhance oxidative metabolism in response to the other major physiological PMN chemoattractants. In addition to GM-CSF, other substances prime PMN, including endotoxin, f-MLP, and a product of B lymphocytes.⁹⁻¹¹ It was also of interest to determine whether the mechanism for priming by GM-CSF was the same as for these other substances.

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peroxide anion production was measured as superoxide dismutase-inhibitable cytochrome C reduction. Purified biosynthetic GM-CSF enhanced superoxide anion production by neutrophils in response to f-MLP, C5a desArg, and LTB₄. In contrast to several other factors which prime neutrophils, GM-CSF did not prime for an enhanced oxidative response to phorbol myristate acetate (PMA). These results suggest that GM-CSF may be an endogenous regulator of neutrophil inflammatory responses induced by the major physiological chemoattractants.

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MATERIALS AND METHODS

Materials. Phorbol myristate acetate (PMA) (Consolidated Midland Corp, Brewster, NY) was dissolved in absolute ethanol, 0.05 mg/mL, aliquoted and stored at -70 °C until used at a final concentration of 0.05 µg/mL. Purified C5a desArg (Upjohn Diagnostics, Kalamazoo, MI) was used at a final concentration of 8 ng/mL. The purity of C5a desArg was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of radioiodinated C5a desArg which was visualized by radioautography. LTB₄ (Calbiochem, La Jolla, CA) was used at a final concentration of 10⁻⁶ mol/L. f-MLP (Sigma Chemical Co, St. Louis) was dissolved in dimethyl sulfoxide and used at a final concentration of 10⁻⁸ mol/L.

Isolation of human PMN. PMN were isolated from heparinized peripheral blood of healthy subjects by Ficoll-Hypaque gradient centrifugation as previously described.^{12,13} Red blood cells (RBC) were removed by sedimentation in 6% dextran (High Fraction, J.T. Baker Chemical Co, Phillipsburg, NJ). Residual RBC were subjected to hypotonic lysis for 30 seconds. The resulting cell preparations containing 97% PMN were washed and resuspended in Hanks' balanced salt solution (HBSS) (without phenol red) containing 10% fetal calf serum (FCS).

GM-CSF. GM-CSF was purified from serum-free medium conditioned by COS monkey kidney cells transfected with the GM-CSF cDNA clone in the p91023(B) vector as described previously, using gel filtration and reverse phase high performance liquid chromatography (HPLC).^{6,8} Purification of GM-CSF to apparent homogeneity was confirmed by the presence of a single iodinated 22,000-dalton protein band by SDS-PAGE. The protein concentration was determined by amino acid analysis; the specific activity of the purified protein assayed on human bone marrow is approximately 2 × 10⁸ units per mg.¹⁴ Purified GM-CSF was shown to be free of measurable endotoxin by the Limulus amoebocyte lysate assay (Sigma Chemical Co, St. Louis). Purified biosynthetic GM-CSF was used at a concentration of 100 pmol/L to study its effect on PMN oxidative metabolism. Maximal stimulation of colony formation by bone marrow cells and KG-1 cells as well as enhancement of neutrophil oxidative metabolism in response to f-MLP is seen at approximately 100 pmol/L.¹⁵

Assay for superoxide anion. PMN (5 × 10⁶/mL) were suspended in HBSS (without phenol red) containing 10% FCS, and 0.4 mL aliquots containing 2 × 10⁶ cells were incubated for two hours at 37 °C with 0.04 mL of protein diluent (negative control) or GM-CSF. After incubation, PMN were equally divided (1 × 10⁶ PMN/tube) and 0.010 mL of superoxide dismutase was added to one tube at a final concentration of 0.02 mg/mL. C5a or LTB₄ were

Table 1. Effect of Purified Recombinant GM-CSF on PMN Oxidative Metabolism

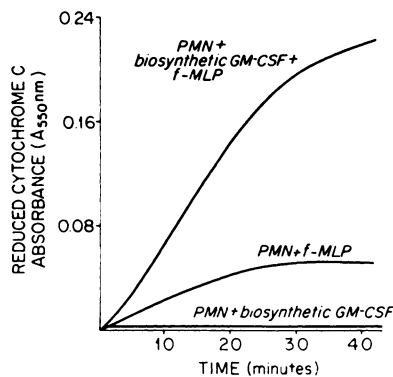
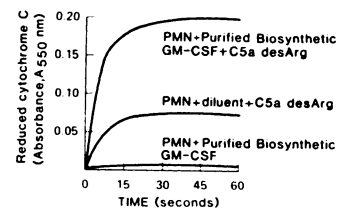
Oxidative Stimulus	Incubation Mixture	No. of Exps	Duration of Experiment (min)	Total Superoxide Anion Produced (nmol)*
f-MLP	diluent	4	4	6.0 ± 0.4
	GM-CSF	4	4	16.0 ± 0.4
C5a desArg	diluent	4	1	1.7 ± 0.1
	GM-CSF	4	1	3.2 ± 0.4
LTB ₄	diluent	4	1	2.4 ± 0.4
	GM-CSF	4	1	4.4 ± 0.4
PMA	diluent	4	25	74.4 ± 3.0
	GM-CSF	4	25	76.8 ± 1.0

*Mean response ± SEM of 10⁶ PMN.

added to each tube, thoroughly mixed, followed by the addition of 1 mL of cytochrome C (1.2 mg/mL in HBSS with 10% FCS). The cell suspensions were transferred to disposable 1-mL cuvettes, and superoxide dismutase-inhibitable cytochrome C reduction was measured spectrophotometrically at 550 nm for one minute at 37 °C with a Varian DMS 90 dual-beam spectrophotometer. Absorbance was recorded continuously with a strip-chart recorder and also at five-second intervals with a computer-assisted program. Superoxide anion production (nmoles) was calculated from reduced cytochrome C using an extinction coefficient of 21,100 mol/L⁻¹ cm⁻¹ and expressed as the mean determination of multiple experiments ± SEM.¹⁶ The effect of GM-CSF on oxidative metabolism of PMN in response to PMA and f-MLP was determined by incubating 2 × 10⁶ PMN with control and test preparations as above, adding 2 mL of cytochrome C, and then equally dividing the cell suspension into separate tubes. Superoxide dismutase was added to one tube, and PMA or f-MLP was added to both tubes. The cells were mixed and immediately transferred to cuvettes where the formation of reduced cytochrome C was monitored for four minutes (f-MLP) or 25 minutes (PMA). The data were recorded and the results calculated as described above.

RESULTS

Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to f-MLP. In preliminary experiments, a dose-response curve was established for f-MLP, and maximum production of superoxide anion occurred at a concentration of 10⁻⁸ mol/L f-MLP. Total superoxide anion produced over four minutes by 10⁶ PMN

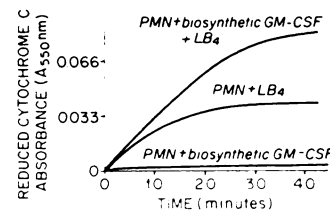
**Fig 1. Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to f-MLP.****Fig 2. Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to C5a desArg.**

incubated with the protein diluent (negative control) was 6.0 ± 0.4 nmoles in response to 10⁻⁸ mol/L f-MLP (mean response of four experiments). Maximum priming by GM-CSF for oxidative metabolism was shown to occur at two hours.⁷ PMN preincubated for two hours with purified recombinant GM-CSF and then stimulated with f-MLP produced an average of 16.0 ± 0.4 nmoles superoxide anion (mean response of four experiments) (Table 1). A representative example of one experiment is shown in Fig 1. Enhancement of a maximal f-MLP response indicates a total increase in PMN oxidative metabolism and excludes measuring a shift in the f-MLP dose-response curve. The purified preparation of GM-CSF was free of measurable endotoxin (<0.1 ng/mL).

Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to C5a desArg. C5a desArg stimulated the production of 1.7 ± 0.1 nmoles superoxide anion over one minute (mean response of four experiments). PMN preincubated with GM-CSF, however, produced 3.2 ± 0.4 nmoles superoxide anion over the same period of time (mean response of four experiments) (Table 1). A representative experiment is shown in Fig 2.

Effect of purified biosynthetic GM-CSF on superoxide anion production in response to LTB₄. Maximum stimulation of oxidative metabolism of PMN in response to LTB₄ occurred at a concentration of 10⁻⁶ mol/L. At this concentration, LTB₄ stimulated the production of 2.4 ± 0.4 nmoles of superoxide anion by 10⁶ PMN over one minute (mean response of four experiments). Preincubation of PMN with GM-CSF for two hours followed by LTB₄ increased superoxide anion production to 4.4 ± 0.4 nmoles (mean response of four experiments) (Table 1). A representative experiment is shown in Fig 3.

Effect of purified biosynthetic GM-CSF on superoxide anion production in response to PMA. The specificity of PMN priming by GM-CSF was examined by measuring the effect of GM-CSF on PMN oxidative metabolism in

**Fig 3. Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to LTB₄.**

response to PMA. In the absence of GM-CSF, 10^6 PMN produced 74.4 ± 3.0 nmoles superoxide anion over 25 minutes which represents the entire course of PMA-induced activation (mean response of four experiments). After preincubating PMN with GM-CSF, the total response was unchanged with 76.8 ± 1.0 nmoles superoxide anion produced (mean response of four experiments) (Table 1). There was also no change in the rate of superoxide anion production. A representative example is shown in Fig 4.

DISCUSSION

PMN are chemoattracted to sites of inflammation where they serve as cytotoxic effector cells. Oxidative metabolism in PMNs is activated by physiological chemoattractants such as bacterial-related f-MLP, serum complement-derived C5a, and the 5-lipoxygenation product of arachidonic acid metabolism, LTB₄, with the production of toxic free radicals of oxygen.²⁻⁵ Products of oxidative metabolism provide one mechanism whereby PMN are cytotoxic.

Lymphokines have been described as endogenous regulators of oxidative metabolism in human monocytes,¹⁷ and colony-stimulating factors have been shown to potentiate granulocyte-mediated cellular cytotoxicity in the murine system.^{18,19} A potential physiological regulator of PMN oxidative metabolism was recently identified as the T cell-derived lymphokine, GM-CSF. GM-CSF, a 22,000-dalton glycoprotein, was shown to enhance the production of superoxide anion by PMN in response to f-MLP.⁷ The present studies were designed to determine whether GM-CSF also regulated PMN oxidative metabolism in response to other physiological chemoattractants.

Our results showed that PMN preincubated with biosynthetic purified GM-CSF enhanced superoxide anion production in response to purified C5a desArg and LTB₄ as well as f-MLP. Each chemoattractant was used at an optimum dose for stimulating superoxide anion production, so enhancement represented an increase in total response and not a shift in the dose-response curve. In contrast to GM-CSF priming for chemoattractants, GM-CSF did not enhance oxidative metabolism in response to PMA. There was no effect on the rate of superoxide anion production or the total amount of superoxide anion produced by PMN during the entire course of PMA activation.

Recently, endotoxin was shown to prime PMN for enhanced oxidative responses to f-MLP.¹⁰ Moreover, the time course for priming was similar to that obtained with GM-CSF. Several observations, however, exclude the possi-

bility that our results were due to the presence of endotoxin. First, HPLC-purified biosynthetic GM-CSF was compared to the HPLC diluent and to the other HPLC fractions that would contain the same contaminants. In addition, endotoxin was not detected by the Limulus amoebocyte assay in HPLC fractions containing neutrophil-activating activity, although the Limulus assay is sensitive enough to detect quantities of endotoxin considerably smaller than those required to prime PMN for enhanced oxidative metabolism.¹⁰ Finally, endotoxin and GM-CSF are different with respect to priming PMN in response to PMA. Endotoxin primes PMN for enhanced oxidative metabolism in response to PMA, whereas GM-CSF does not.

Lipoxygenase products also enhance neutrophil oxidative metabolism in response to f-MLP, but their effect of PMA responses has not been examined.²⁰ It will be of interest to compare the mechanism of action of these products with GM-CSF since these products could be responsible for the GM-CSF effect.

Other substances have also been shown to prime PMN for enhanced oxidative metabolism. In addition to endotoxin, priming has been shown to occur in response to subthreshold concentrations of f-MLP.^{9,21} Moreover, priming occurs in response to a factor present in the supernatant of a B lymphoblast cell line.¹¹ In contrast to GM-CSF, however, f-MLP, the B cell-derived factor, and endotoxin all prime PMN for enhanced oxidative metabolism in response to PMA.^{9,11,22,23} This finding suggests multiple mechanisms for priming with some specificity in the mechanism for PMN priming by GM-CSF. Although the mechanism for PMN priming by GM-CSF is unknown, a variety of secretory stimuli have been shown to increase f-MLP receptor availability by mobilizing an intracellular pool of f-MLP receptors.¹⁶ Moreover, recent studies show that short (15-minute) incubations of GM-CSF with neutrophils resulted in increased expression of high-affinity f-MLP receptors corresponding with enhanced chemotaxis in response to f-MLP, whereas more prolonged incubation of neutrophils with GM-CSF (two hours) resulted in the development of low-affinity receptors corresponding with enhanced oxidative metabolism in response to f-MLP.²⁴

PMN oxidative metabolism provides a mechanism for host defense through the production of potent derivatives of oxygen with microbicidal and tumoricidal activity. PMN oxidative metabolism requires precise regulation and control, as products of oxidative metabolism may induce tissue injury as well. GM-CSF may be an endogenous regulator of PMN oxidative metabolism and contribute to host defense-related reactions. In addition, GM-CSF could play a role in the pathophysiology of tissue injury associated with excessive inflammation as occurs in autoimmune diseases. GM-CSF regulation of PMN oxidative metabolism in response to different physiological chemoattractants may provide a common basis for understanding diverse biological functions of GM-CSF.

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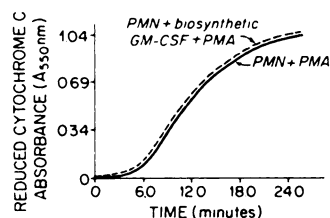


Fig 4. Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to PMA.

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