Human Granulocyte-Macrophage Colony Stimulating Factor: An Effective Direct Activator of Human Polymorphonuclear Neutrophilic Granulocytes

Alexander Kapp, M.D., Gabriele Zeck-Kapp, Ph.D., Martin Danner, Ph.D., and Thomas A. Luger, M.D.
Department of Dermatology and Department of Pathology, University of Freiburg, F.R.G. (AK, GK); and Second Department of Dermatology and LBI for dermatovenerological serodiagnosis, Laboratory for cell-biology, University of Vienna, Austria (MD, TAL)

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to modulate different granulocyte functions. In the present study we investigated the effect of purified and recombinant human GM-CSF, particularly on the oxidative metabolism of isolated human granulocytes. In addition, ultrastructural changes upon stimulation were evaluated. For detection of granulocyte activation the following assay systems were used: 1) lucigenin-dependent chemiluminescence (CL), 2) superoxide-dismutase (SOD) inhibitable cytochrome C-reduction (superoxide), 3) horseradish peroxidase-mediated oxidation of phenol red (hydrogen peroxide), 4) release of myeloperoxidase, 5) ultrastructural detection of hydrogen peroxide-production, and 6) scanning and transmission electron microscopy (SEM and TEM, respectively). A significant CL response was seen upon stimulation with recombinant human GM-CSF at concentrations ranging from 1 to 10^3 U/ml. The CL response started within 5–10 min with a maximum at 60–90 min and lasted more than 3 h. Thereafter granulocytes were completely deactivated to restimulation with the same mediator and with Tumor Necrosis Factor, but responded to other triggers of the oxidative burst, whereas the response to f-met-leu-phe was significantly increased. The CL signal was completely blocked by an antiserum to GM-CSF. Moreover, the response was significantly inhibited by SOD and D-Mannitol, suggesting the involvement of distinct reactive oxygen species (ROS) in generating the CL response. Significant amounts of superoxide were detected within 180 min after stimulation with GM-CSF, whereas release of hydrogen peroxide and peroxidase were only minimal as shown by functional and ultrastructural assays. Activation of granulocytes could be visualized by SEM and TEM. GM-CSF stimulated cells showed an increased adherence to the substratum developing polarized filopodia and an increased number of intracellular vesicles within 30 min after addition of the stimulus. The results clearly demonstrate that GM-CSF directly stimulates granulocytes and, particularly, their oxidative metabolism. Therefore, GM-CSF which is probably released by epidermal cells appears to be a candidate for neutrophil activation in the skin, and thereby may play a crucial role in inflammatory skin diseases. *J Invest Dermatol 91:49–55, 1988*

Colony-stimulating factors are a group of glycoprotein hormonal growth factors that sustain the process of blood cell formation by controlling proliferation and differentiation of precursors of hematopoietic cells [1]. Similar to other cytokines, CSFs are not only released by immunocompetent cells [2–5]. Accordingly, GM-CSF, which is believed to be identical with neutrophil migration inhibition factor from T-lymphocytes (NIF-T) [6], recently has been shown to be generated by epidermal cells [7–9]. Moreover, this cytokine seems to be related to a previously described epidermal cell granulocyte-activating mediator (EC-GRAM), because anti-GM-CSF antibodies were capable of blocking EC-GRAM activity [10]. Highly purified natural or recombinant human GM-CSF stimulates granulocyte/macrophage and eosinophil colony formation in vitro. In addition to its effect on progenitor cells, GM-CSF also induces a variety of functional changes in mature cells. Particularly, GM-CSF was demonstrated to modulate different PMN functions specifically following binding to its receptor [11]. It primes human PMN for enhanced oxidative metabolism in response to chemotactic stimuli, such as f-met-leu-phe, probably by modulation of receptor number and affinity [12–13]. Furthermore, GM-CSF increases antibody-dependent cytotoxicity, phagocytosis, and killing of pathogenic microbial organisms and parasites [14–16]. We therefore investigated whether human GM-CSF is capable of directly activating the oxidative metabolism of human granulocytes by means of functional and morphological criteria.

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Reprint requests to: Dr. Alexander Kapp, Hautklinik der Universität, Hauptstrasse 7, D-7800 Freiburg, F.R.G.

Abbreviations:
- BSA: Bovine serum albumin
- CL: Chemiluminescence
- CSF: Colony stimulating factor
- GM-CSF: Granulocyte-Macrophage colony stimulating factor
- LPS: Bacterial lipopolysaccharide
- MNC: Mononuclear leukocytes
- PMN: Polymorphonuclear neutrophilic granulocytes
- SEM: Scanning electron microscopy
- TEM: Transmission electron microscopy
- TNF: Tumor necrosis factor

MATERIALS AND METHODS

Cytokines Purified human GM-CSF (CSF-H) was purchased from Genzyme (Boston, MA) and was produced by a HTLV-I

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virus-transformed human leukemic T-cell line. Originally purified by ion-exchange chromatography, this material was devoid of interleukin 1, interleukin 2, and interferon activities and was free of endotoxin or inducing agents. Its specific activity was 2200 CFUc/μg.

Escherichia coli-derived recombinant human GM-CSF [14] was a kind gift from Dr. J. DeLamarter (Biogen, Geneva, Switzerland) and had a specific activity of 1.1×10^5 U/μg in the CML assay [17]. The material had an endotoxin concentration of 0.5 EU/10 μg in the Limulus amoebocyte lysate assay. Recombinant human TNF/Cachectin was a kind gift from Dr. D. Blohm (BASF, Ludwigshafen, F.R.G.). Recombinant human G-CSF and M-CSF/CSF-1 were a kind gift from Dr. Seiler (Behring, Marburg, F.R.G.). Recombinant human Interleukin-3 and Interleukin-4/BSF-1 were purchased from Genzyme (Boston, MA). Before testing all cytokines were diluted in PBS containing 50 μg/ml BSA.

Reagents and antibodies: Ficoll-Paque solution was obtained from Pharmacia (Freiburg, F.R.G.); purified bovine serum albumin (BSA), lucigenin, phorbol-myristate-acetate (PMA), f-met-leu-phe, zymosan A, ferricytochrome C (type III), cytochalasin B, phenolsulfonphtalein (phenol red, sodium salt), D-mannitol, dimethylthiourea (DMTU), o-phenylen-diamine (OPD), cetyl-trimethylammonium bromide (CETAB), horseradish peroxidase (HRPO, type II), superoxide dismutase (SOD, type I), catalase (bovine liver), and normal rabbit and calf serum were obtained from Sigma (Munich, F.R.G.). Culture media and antibiotics were purchased from Biochrom-Seromed (Berlin, F.R.G.). $CeCl_3$ and 3-Amino-1,2,4 triazole (AT) were from Fluka (Buchs, Switzerland). Reagents and media for electron microscopy were obtained from Roth (Karlsruhe, F.R.G.). A calf and a rabbit antiserum to recombinant human GM-CSF were a gift from Dr. P. Moonen (Biogen, Geneva, Switzerland). A rabbit antiserum to recombinant human TNF/Cachectin was kindly provided from Dr. A. Möller and Dr. D. Blohm (BASF, Ludwigshafen, F.R.G.). Opsonized zymosan was prepared as described previously [18].

Isolation of human PMN Human PMN were isolated from heparin-anticoagulated venous blood of healthy blood donors (as described in Ref 10) by ficoll gradient centrifugation and three 30 sec cycles of 0.2% NaCl treatment, followed by the addition of an equal volume of 1.6% NaCl to lyse red blood cells. Cells were 98% polymorphonuclear leucocytes as judged by Pappenheim stain and more than 95% viable by trypan blue exclusion. Subsequently, PMN were suspended in different media as indicated below.

Lucigenin-dependent chemiluminescence (CL) Lucigenin-dependent CL [10,19], which, in contrast to the luminol enhanced reaction, is independent from the release of peroxidase [20], represents an extremely sensitive measure of the release of reactive oxygen species (ROS) from PMN. However, the CL response is not known to be strongly correlated with the production of a particular ROS. Subsequently, for CL measurements PMN were suspended to a density of 5×10^6 cells/ml in HEPES-buffered Hank's balanced salt solution (HBSS), pH 7.4, containing 2 mM Lucigenin and 1 mg/ml BSA. Aliquots (200 μl), containing 1×10^6 cells each, were placed into unsealed polystyrene luminescence tubes (Lumacuvette/Abimed, Düsseldorf, F.R.G.) and stored at 4°C for a maximum of 6 h prior to use. Measurements were performed in a six channel Biolumat LB 9505 (Berthold, Wildbad, F.R.G.) interfaced via an Apple II E computer to an Epson RX80 graphic printer. The thermostat was set at 37°C throughout. 50 μl of the stimulus were added to the PMN corresponding an 1:5 dilution. Integral counts from a 0-to-60-min incubation interval were measured after addition of stimuli to the granulocytes and are indicated as counts in Figures and Tables.

Detection of superoxide production Detection of superoxide was performed by measurement of the SOD-inhibitable part of cytochrome C reduction in a modification of the micro-assay described by Pick and Mizel [21]. In brief, PMN at a concentration of 10×10^6/ml were resuspended in HBSS, containing 1 mg/ml BSA. Aliquots of 100 μl were distributed to 96-well flat-bottom tissue culture plates. Subsequently cells were covered with cytochrome C at a final concentration of 120 μM, in the presence or absence of SOD (200 μg/ml), and the stimuli or Medium were added. Plates were incubated at 37°C and shaken slightly on a Titertek microplate shaker. The absorbance was measured at 550 nm at the time intervals indicated in a Titertek Multiscan MCC micro ELISA reader (Flow, Bonn, F.R.G.). To compensate for changes in absorbance due to the presence of cells the reference wavelength was set at 492 nm [22]. The results were expressed in nM cytochrome C reduced (= nM $\cdot O_2^-$) per 1×10^6 PMN per well after subtraction of absorbance readings for wells containing SOD.

Detection of hydrogen peroxide production Release of hydrogen peroxide was detected by measurement of the HRPO-mediated oxidation of phenol red in a modification of the micro assay described by Pick and Mizel [21]. In brief, PMN were suspended at a concentration of 5×10^6 /ml in PBS, containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+}. Aliquots of 25 μl were distributed to 96-well flat-bottom tissue culture plates. Cells were covered with a solution containing 2.2 mM Dextrose, 80 μg/ml phenol red, and 8 U/ml HRPO (final concentration) in PBS (pH 7.0) and the stimuli or medium were added. Plates were incubated at 37°C for 60 or 180 min. After the incubation period wells were brought to alkaline pH by addition of NaOH to a final concentration of 70 mM in order to eliminate changes in the absorbance of phenol red due to its behavior as a pH indicator. For blanking, initial absorbance was measured in wells containing the reaction mixture, to which NaOH was added at time 0. For expression of results in terms of nM/ml hydrogen peroxide a standard curve was prepared with H_2O_2 concentrations ranging from 1 to 50 nM/ml [23]. Plates were read in the Titertek Multiscan MCC, using a 600 nm filter. The concentration of hydrogen peroxide released was calculated from the standard curve using a computer-assisted program and expressed as nM/ml released by 1.25×10^5 PMN per time interval.

Release of Peroxidase Myeloperoxidase [24] was measured as described previously by Pember and Kinkade [25] in a modification of the method used by Schröder [26]. Briefly, PMN were suspended at a concentration of 5×10^6/ml in PBS containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+}. Assays were performed in 96-well flat bottom tissue culture plates in the presence of 5 μg/ml cytochalasin B using 5×10^5 cells/well. After addition of the stimulus PMN were incubated for 60 min at 37°C, shaken slightly on a Titertek microplate shaker, and centrifuged immediately. Total cellular peroxidase activity was determined by addition of CETAB to the PMN at a final concentration of 0.4%.

Supernatants were transferred and indicator reagents were added. The final reaction mixture consisted of 0.5 mg/ml OPD, 0.1 mM hydrogen peroxide, and 0.1% CETAB in Phosphate-Citrate buffer (pH 5.5). The reaction was stopped by acidification; the reaction product was measured at 492 nm in a Titertek Multiscan MCC. Results were expressed as percent of total content.

Additional Bioassays Samples were tested for chemotactic activity by measuring cell migration with the double chamber filter method of Boyden using blind well chambers as described previously [18]. GM-CSF activity was measured by ^{3}H-thymidine incorporation by human peripheral blood myeloblast cells (CML) as described [27].

High performance liquid chromatography (HPLC) HPLC was performed by a system supplied from LKB (Bromma, Sweden) as described [28]. One hundred microliter samples were subjected to reversed phase HPLC using a RP-304 high pore, 250 × 4.5 mm (Bio-Rad) column that was equilibrated with distilled water. Elution was carried out by applying an acetonitrile gradient ranging from 0%–100%, at a flow rate of 1.5 ml/min. Column fractions were lyophilized, reconstituted in PBS containing BSA, and sterile filtered.

Ultrastructural detection of granulocyte activation For evaluation of the effect of GM-CSF by ultrastructural criteria PMN were incubated at a concentration of 5×10^6/ml in microtubes and 1×10^6/ml in Leighton tubes (Costar, Fernwald, F.R.G.) for transmission and scanning electron microscopy, respectively (TEM and SEM). Thereafter, PMN were stimulated with GM-CSF (10, 10^2, and 10^3 U/ml) for 15 and 30 min at 37°C or remained unstimulated; PMA (10 ng/ml) served as a control stimulus. After the incubation period PMN were fixed immediately for TEM or SEM as described below or they were subjected to hydrogen peroxide detection and subsequently fixed for TEM.

Hydrogen peroxide production was detected as described by Briggs et al [29]. Briefly, after centrifugation unfixed cells were preincubated for 10 min at 37°C in 0.1 M Tris-Maleate with 7% sucrose (pH 7.5) containing 1 mM AT. The final incubation medium consisted of 0.1 M Tris-Maleate with 7% sucrose (pH 7.5), 10 mM AT, and 1 mM $CeCl_3$. PMN were incubated for 30 min at 37°C in this medium. Thereafter, PMN were briefly washed in Tris-maleate buffer with 7% sucrose at 4°C and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) with 5% sucrose for 60 min at 4°C. In the cerium precipitation series cells were subsequently washed in sodium cacodylate buffer (pH 6.0) with 5% sucrose for 60 min at 4°C. Generally, PMN were washed over night in 0.1 M sodium cacodylate buffer (pH 7.3) with 5% sucrose, postfixed in 2% osmium tetroxide in the same buffer for 60 min, dehydrated in graded ethanols, and embedded in Araldit. Thin sections were routinely stained with 4% aqueous uranyl acetate and lead citrate and examined in a Zeiss EM 109 R.

For SEM, PMN were fixed in glutaraldehyde and postfixed in osmium tetroxide as described above. Samples were dehydrated in a graded series of acetone (30–100%), critical-point dried, coated, and examined in a Jeol JSM-35CF scanning microscope.

Statistical analysis Statistical significance of the data was calculated using Student's t-test.

RESULTS

Characterization of the CL response Purified preparations of native human GM-CSF and recombinant human GM-CSF both were able to induce a significant CL response (Fig 1*A*,*B*). Recombinant GM-CSF stimulated PMN even at a concentration of 1 U/ml and showed a dose related response up to 10^3 U/ml, whereas higher concentrations did not enhance the CL signal (Fig 1*A*). Testing the effect of purified GM-CSF gave comparable results in the range of 1 to 100 U/ml (Fig 1*B*). However, increasing the concentration of purified GM-CSF up to 10^3 U/ml resulted in a significantly decreased CL response. Native and recombinant GM-CSF induced a long-lasting and intense CL signal. CL response was observed within 5–10 min, showing a maximum at 60–90 min (Fig 2*A*,*B*). Integral counts within 180 min induced by GM-CSF (10^2 – 10^3 U/ml) were comparable to the effect of PMA (10 ng/ml). At high concentrations of GM-CSF, a double-peaked CL signal was seen (Fig 2*A*), whereas at lower concentrations the first peak disappeared (Fig 2*B*). Activation lasted more than 180 min. Thereafter, PMN were completely deactivated to restimulation with the same mediator and with TNF (Table I). However, PMN responded (to a lower degree) to stimulation with PMA and opsonized zymosan. In contrast, the response to the chemotactic peptide f-met-leu-phe was significantly increased after preincubation with GM-CSF (Fig 3).

To further clarify the specificity of the CL response, GM-CSF preparations were subjected to RP-HPLC and the fractions were tested for activity in the CL and CML assay. Purified and recombinant GM-CSF eluted on RP-HPLC at 40%–50% acetonitrile exhibiting GM-CSF activity when tested for their capacity to enhance the proliferation of human chronic myeloid leukemia cells, as well as the capability to induce a CL response (Data not shown). Furthermore, the CL signal induced by GM-CSF was completely blocked by a calf antiserum [Table II(A)] and a rabbit antiserum (Data not shown) against recombinant human GM-CSF, whereas the response induced by TNF was unaffected. In addition, the effect induced by GM-CSF was unaffected by a rabbit antiserum against recombinant human TNF/Cachectin [Table II(B)]. In order to investigate the relationship of GM-CSF to other cytokines with CSF activity, recombinant human G-CSF, M-CSF, Interleukin-3, and Interleukin-4 were tested for their capability to induce a CL response in human granulocytes. G-CSF, M-CSF (10 to 10^5 U/ml), and Interleukin-4 (10 to 4×10^3 U/ml) did not show a significant effect on human PMN. Only Interleukin-3 elicited a distinct CL

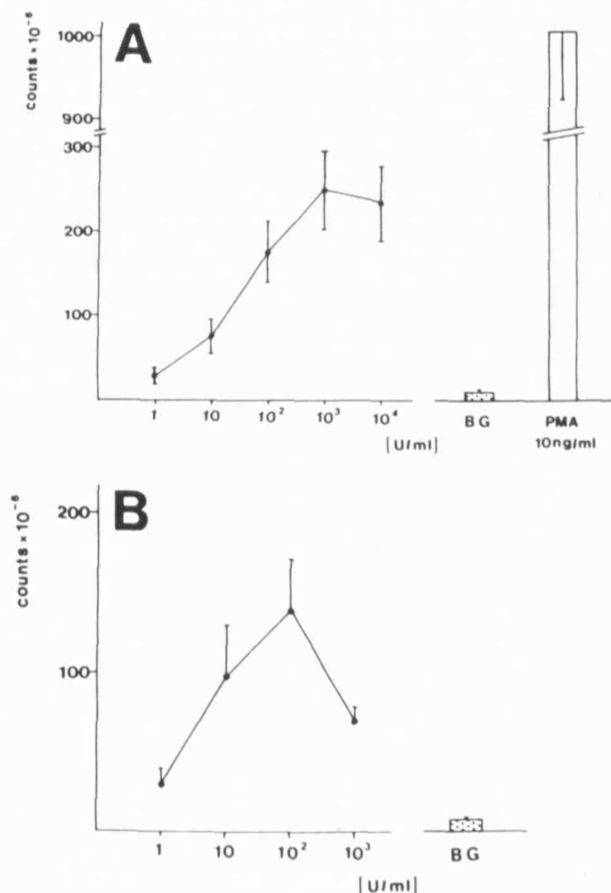

Figure 1. *A*) CL response induced by recombinant human GM-CSF. Values represent the mean ± SEM of 7 experiments on 5 donors. *B*) CL response induced by purified human GM-CSF. Values represent the mean ± SEM of 6 experiments on 4 donors. CL response induced by PMA (10 ng/ml) was 1048 ± 92. 60 min integral counts were measured. *BG*: Background activity induced by addition of medium.

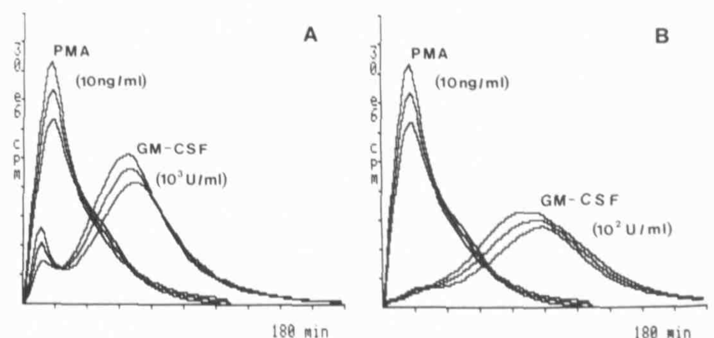

Figure 2. Time course of the CL response induced by 10^3 U/ml (*A*) and 10^2 U/ml (*B*) recombinant human GM-CSF. PMA (10 ng/ml) served as a positive control. Values represent the mean ± SE of triplicate measurements.

Table I. Effect of Preincubation with GM-CSF on the CL Response of PMN to Stimulation with Different Stimuli.

Stimulus	Preincubation with[a] GM-CSF	Medium	
Medium	25.5 ± 2.9[b]	34.3 ± 8.0	N.S.[c]
GM-CSF (10^3 U/ml)	28.1 ± 5.0	155.6 ± 45.4	$p < 0.05$
TNF (10^3 U/ml)	48.3 ± 9.4	282.7 ± 71.3	$p < 0.05$
PMA (10 ng/ml)	398.1 ± 76.0	816.6 ± 174.4	N.S.
opsonized zymosan (1 mg/ml)	680.6 ± 69.9	1070.8 ± 110.4	$p < 0.05$

[a] PMN were preincubated with 10^3 U/ml recombinant human GM-CSF or medium for 180 min at 37°C and subsequently stimulated with the stimuli indicated.
[b] Values are expressed as 60 min integral counts (counts × 10^{-6}) and represent the mean ± SEM of 4 experiments on 4 donors.
[c] Not significant

signal at a concentration of 10^3 U/ml, whereas lower concentrations were ineffective (Data not shown).

To further characterize the source of ROS responsible for the CL signal induced by GM-CSF the effect of different scavengers of ROS was tested: SOD ($\cdot O_2^-$), Catalase (H_2O_2), D-mannitol, and DMTU [30] ($\cdot$OH) (Fig 4). The signal was significantly inhibited by SOD and D-mannitol, and to a lower degree by DMTU. Catalase was ineffective in blocking the CL response.

Production of superoxide and hydrogen-peroxide, release of myeloperoxidase and chemotaxis Release of superoxide upon stimulation with GM-CSF was detected at concentrations of 10 to 10^3 U/ml (Fig 5). At higher concentrations no significant increase of superoxide production was observed. Detectable generation of superoxide started dose-related in the range of 30-120 min after addition of the stimulus and reached a maximum within 180 min. This stimulation pattern was in contrast to effects induced by the control stimulus PMA, showing maximal cytochrome C-reduction within 30 min.

Release of H_2O_2 in the supernatant following stimulation with GM-CSF was minimal even at a concentration of 10^4 U/ml GM-CSF for 180 min (Fig 6). Lower concentrations of GM-CSF and/or shorter incubation periods (60 min) did not result in significant production of hydrogen-peroxide.

GM-CSF did not induce significant release of myeloperoxidase

Table II. Effect of Antisera Against GM-CSF and TNF on the CL Response of PMN to Stimulation with GM-CSF and TNF.

A Stimulus	CL response (counts × 19^{-6})[a] + calf serum[b]	+ calf anti-GM-CSF	
Medium	26.1 ± 3.2	25.9 ± 3.5	N.S.[d]
GM-CSF	166.8 ± 26.4[c]	32.1 ± 4.0	$p < 0.001$
TNF	683.8 ± 106.5	554.3 ± 58.1	N.S.

B Stimulus	CL response (counts × 10^{-6})[a] + rabbit serum[b]	+ rabbit anti-TNF	
Medium	14.3 ± 2.2	10.0 ± 0.4	N.S.[d]
GM-CSF	206.2 ± 17.2[c]	176.0 ± 27.5	N.S.
TNF	311.6 ± 24.7	23.3 ± 7.7	$p < 0.001$

[a] 60 min integral counts were measured.
[b] 30 μl recombinant human GM-CSF (10^4 U/ml), TNF (10^4 U/ml), or Medium were incubated with 30 μl normal calf serum/rabbit serum or calf antiserum to recombinant human GM-CSF (1:10)/rabbit antiserum to recombinant human TNF/Cachectin (1:20) for 30 min at room temperature; subsequently, 50 μl were added to the PMN for testing the CL response.
[c] Values represent the mean ± SEM of 4-6 experiments.
[d] Not significant.

(Table III) and was devoid of chemotactic activity in the range of 1 to 10^3 U/ml (Data not shown).

Ultrastructural Changes and Localization of Hydrogen Peroxide Production Nonstimulated cells remained rounded with ruffled surface membranes during incubation (Fig 7*A*) showing the characteristic morphology of PMN which includes a multilobulated nucleus, a large population of morphologically diverse granules, few mitochondria, and scattered deposits of glycogen (Fig 7*B*). Treated cells, still rounded after incubation with 10 U/ml GM-CSF for 15 min, began to spread and to extrude numerous filamentous filopodia which elongated with time (30 min). Upon stimulation with 10^2 or 10^3 U/ml GM-CSF for 15 min, PMN adhered more firmly, exhibiting a long dendritic protrusion mainly seen on only one side of the cells. After an incubation period of 30 min, PMN appeared to be flattened and were typically polarized (Fig 7*C*). Intracellular alterations were evident in the cytoplasma of the PMN within 15 min after addition of 10 U/ml GM-CSF, showing an increased number of intracellular vesicles. At higher

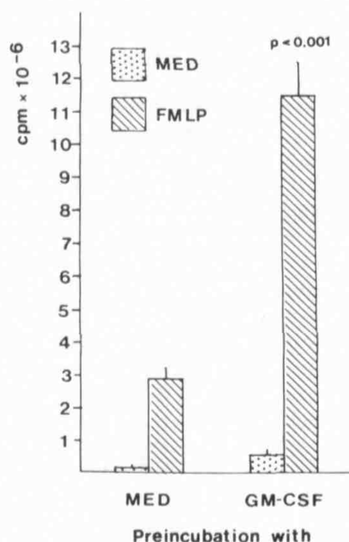

Figure 3. Effect of preincubation with recombinant human GM-CSF on the CL response induced by the chemotactic peptide f-met-leu-phe. PMN were preincubated with 10^4 U/ml recombinant human GM-CSF or medium (MED) for 150 min; subsequently, medium (MED) or f-met-leu-phe at a final concentration of 10^{-6} M were added and the peak CL within 15 min was measured. Values represent maximal counts per minute within 15 min after addition of the stimulus of 11 experiments on 4 donors. $p < 0.001$ vs. preincubation with Medium.

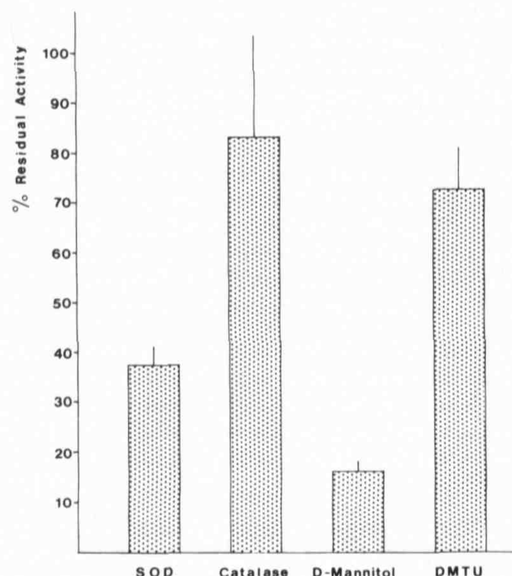

Figure 4. Effect of scavengers of reactive oxygen species on the CL signal induced by GM-CSF. PMN were stimulated with 10^3 U/ml recombinant human GM-CSF in absence (=100%) or presence of SOD (200 μg/ml), Catalase (200 μg/ml), D-Mannitol (100 mM), and DMTU (10 mM). 60 min integral counts were measured. Values were expressed as per cent residual activity and represent the mean ± SEM of 3 experiments on 2 donors.

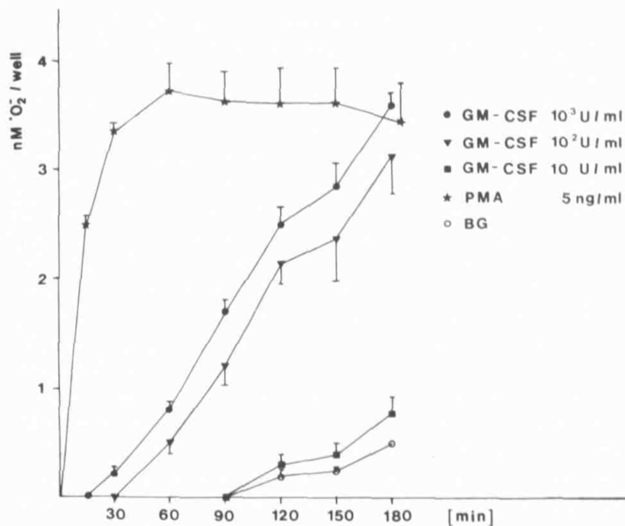

Figure 5. Production of superoxide induced by recombinant human GM-CSF. PMN were stimulated with different concentrations of GM-CSF and the SOD-inhibitable part of cytochrome-C reduction was measured. *BG:* Medium was added instead of GM-CSF. Values represent the mean ± SEM of 3 experiments on 3 donors.

concentrations of GM-CSF (10^2-10^3 U/ml) vesicles were larger in size and more abundant. Similar results were obtained following stimulation with 10 U/ml GM-CSF for 30 min (Fig 7*D*). Formation of large vesicles was associated with a decrease in number of granules present in the cytoplasma. Upon stimulation with PMA (10 ng/ml), a known activator of PMN, for 15 min the attached cells were adhered firmly to the plastic layer, short filopodia were only seen in the rounded central portion of the PMN, and large vesicles could be detected (Fig 7 *E,F*).

Production of hydrogen peroxide was visualized by ultrastructural detection of the electron-dense precipitate of cerium perhydroxide. After stimulation of the PMN with GM-CSF (10^2-10^3 U/ml) for 30 min the reaction product was found in small intracytoplasmic vesicles, but to a significantly lower degree as compared with PMA-stimulated cells (Fig 7 G,H). In contrast, there was no difference between nonstimulated cells and cells treated with GM-CSF for 15 min. After stimulation with PMA for 15 min the external surface of the plasma membrane and the luminal part of the small intracytoplasmic vesicles were coated with a layer of electron-dense material. No other cytoplasmic organelles were reactive for hydrogen peroxide (Fig 7*H*).

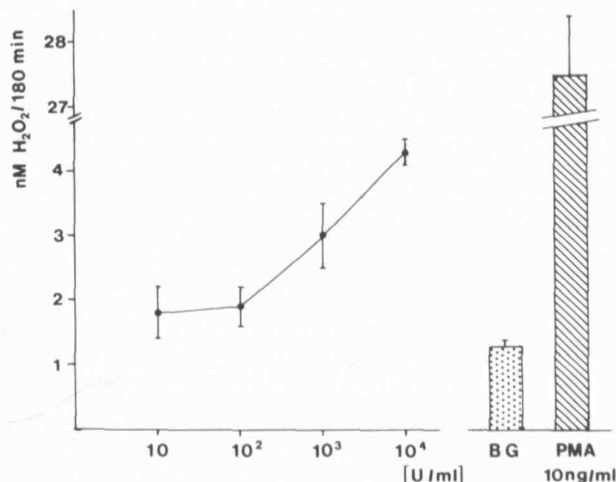

Figure 6. Production of hydrogen peroxide induced by recombinant human GM-CSF. PMN were stimulated with different concentrations of GM-CSF and the HRPO-mediated oxidation of Phenol red was measured for 180 min. BG: Medium was added instead of GM-CSF. Values represent the mean ± SEM of 3 experiments on 3 donors.

Table III. Release of Myeloperoxidase from PMN Stimulated with GM-CSF.

Stimulus	% of total content
GM-CSF (10^3 U/ml)	6.7 ± 3.0[a]
f-met-leu-phe (10^{-7} M)	41.5 ± 6.7
Medium	2.8 ± 1.1

[a] PMN were incubated with the stimuli indicated for 60 min at 37°C in the presence of cytochalasin B and the release of peroxidase was measured as described in the "Methods" section. Values represent the mean ± SEM of 5 experiments on 5 donors.

DISCUSSION

Functional assays and ultrastructural criteria showed that purified native and recombinant human GM-CSF directly activate the oxidative metabolism of human granulocytes, whereas human Inter-

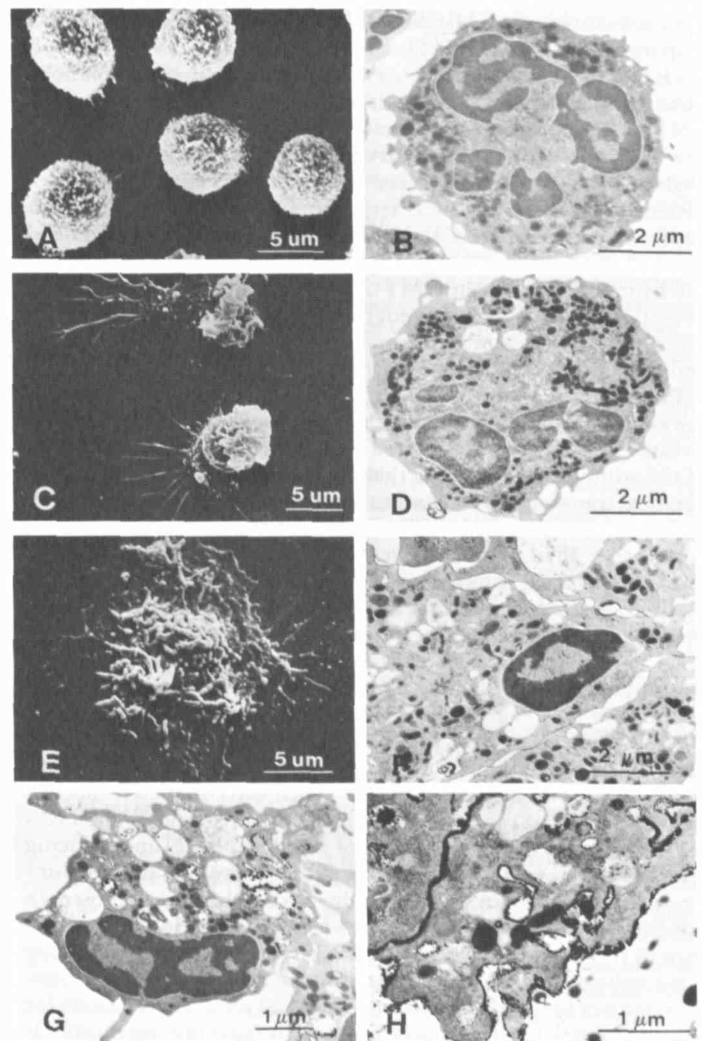

Figure 7. Ultrastructural detection of granulocyte activation induced by recombinant human GM-CSF. *A,* Nonstimulated PMN, 30 min/37°C, SEM; *B,* Nonstimulated PMN, 30 min/37°C, TEM; *C,* PMN stimulated with recombinant human GM-CSF (10^3 U/ml), 30 min/37°C, SEM; *D,* PMN stimulated with recombinant human GM-CSF (10 U/ml), 30 min/37°C, TEM; *E,* PMN stimulated with PMA (10 ng/ml), 15 min/37°C, SEM; *F,* PMN stimulated with PMA (10 ng/ml), 15 min/37°C, TEM; *G,* Ultrastructural detection of hydrogen-peroxide production following stimulation of PMN with recombinant human GM-CSF (10^3 U/ml), 30 min/37°C, TEM; and *H,* Ultrastructural detection of hydrogen-peroxide production following stimulation of PMN with PMA (10 ng/ml), 15 min/37°C, TEM.

leukin 3, Interleukin 4, G-CSF [31,32], and M-CSF [33,34] did not exhibit a significant direct stimulatory effect on human PMN. However, the latter factors were able to prime PMN [32] and macrophages [34], respectively, for an enhanced oxidative response to stimulation. In contrast to previous reports [12,14,15], our data demonstrate that GM-CSF induces a significant superoxide production. However, there were important differences in the assay conditions. Measuring superoxide production we found a dose-related increase in the range of 30 to 120 min after addition of the stimulus. We therefore evaluated superoxide production at different times for a longer incubation period. Furthermore, under the assay conditions described, PMN were adherent to the microplates and were not kept in suspension. In addition, measurement of the SOD-inhibitable part of cytochrome C reduction usually underestimates superoxide production because other ROS released, such as hydrogen peroxide and hydroxyl radical, could cause oxidation of cytochrome C [35]. Thus, we used different techniques to evaluate the effect of GM-CSF on ROS production of PMN. The lucigenin-dependent CL [10,19] represents a highly sensitive measure of the production of oxigenation products, particularly superoxide. Purified native as well as recombinant GM-CSF induced a significant dose-related CL response in human PMN. The response started after a lag phase of 5–10 min which was correlated with the detection of morphologic changes in SEM and TEM reflecting the metabolic activation of PMN. The CL signal was biphasic. This phenomenon could probably be the result of a complex activation process involving more than one step or the generation of disinct ROS and their reaction products. However, the CL response was not simply a result of superoxide because the CL signal could be inhibited by SOD and, particularly, D-mannitol. The latter is suggested to be a scavenger of the hydroxyl radical, but DMTU, which was also reported to be a potent hydroxyl radical scavenger, did not significantly block the CL response. Therefore, distinct ROS are probably involved in generating the complex CL reaction induced by GM-CSF.

Prestimulation of PMN with GM-CSF completely deactivated the cells to restimulation with the same mediator and with TNF. Because PMN were not activated even at the highest concentrations of the stimuli it is suggested that these mediators probably trigger identical transductional events in their targets. The responses (after preincubation with GM-CSF) to different triggers of the oxidative burst other than cytokines were reduced but still at significant levels. In contrast, stimulation with the chemotactic peptide f-met-leu-phe resulted in a significantly enhanced CL signal, a finding that has been reported previously for superoxide production [12,13]. This priming effect was described to be specific for the major physiologic chemoattractants [12]. Therefore, GM-CSF appears not only to be an activator of PMN oxidative metabolism, but an endogenous regulator of the inflammatory response. Specificity of the CL response following stimulation with GM-CSF was confirmed by two criteria: 1) the CL signal was completely blocked by antisera against recombinant human GM-CSF; and 2) upon RP-HPLC fractions that were capable of activating PMN and inducing proliferation of CML cells were identical. Therefore, it is very unlikely that the effect measured was due to contaminations present in the GM-CSF preparations. As shown by SEM techniques, adherence to the plastic surface within 15 min appeared to be a primary event induced by GM-CSF. Upon stimulation with GM-CSF granulocytes were typically polarized within 30 min. These results are in agreement with the effect of GM-CSF on granulocyte morphology described by Lopez et al [15] who noted a change in the shape of granulocytes at 15 min after addition of the stimulus. Therefore, it appears very likely that GM-CSF, which increases the adherence of PMN, does not affect their motility. Moreover, GM-CSF is thought to be identical to NIF-T, the lymphokine that acts to localize granulocytes [6]. However, it has been reported that GM-CSF is a very poor stimulus at promoting adherence of neutrophils to plastic by means of a functional assay [15]. These results are in contrast to the significant morphologic alterations of PMN, indicating enhanced adherence to the plastic layer even at low concentrations of the stimulus. Furthermore, GM-CSF has been reported to increase cell-to-cell adhesion and surface expression of Mo1 and LeuM5 (P150,95), two members of a family of leukocyte adhesion molecules (Leu-CAM) whereby increased Mo1 surface expression occurred within 15 min [36]. Based on our results we suggest that GM-CSF induces the release of ROS by promotion of adherence.

The effect of GM-CSF on PMN was rapid because first intracellular alterations were seen within 15 min after addition of the stimulus. These intracellular signs of activation preceeded the oxidative response, which could be visualized by detection of hydrogen peroxide within 30 min. The reaction pattern indicated the GM-CSF induced activation of a membrane-bound oxidase which was localized on the external surface of the plasma membrane and was due to invagination at the luminal part of the intracytoplasmic vesicles. However, detectable release of hydrogen peroxide in the supernatant, even at high concentrations of GM-CSF, was only minimal. Because, supposedly, distinct ROS are generated simultaneously and react together to form additional oxygenation products, and these reactions take place in zones of close cell-to-cell contact, it is obviously difficult to measure hydrogen peroxide by conventional assays.

A decrease in number of intracellular granules of PMN following stimulation could be detected by TEM. However, the release of myeloperoxidase upon stimulation with GM-CSF was only minimal. Similar results were observed with lysozyme secretion [14,15]. Both enzymes are contained in the azurophilic granules of PMN [24]. Myeloperoxidase represents a component of the myeloperoxidase-halide system which appears to be the most important mechanism enabling PMN to kill bacteria and parasites. GM-CSF did not promote degranulation, but was reported to prime PMN for enhanced release of lysozyme upon stimulation with f-met-leu-phe [14,15].

Taken together, our data demonstrate that GM-CSF is an important activator of granulocyte oxidative metabolism and is capable of modulating specifically distinct metabolic functions. Apart from these findings GM-CSF appears to be the only colony-stimulating factor that directly activates human granulocytes. As reported previously [7-9] GM-CSF is generated by epidermal cells. Furthermore, Interleukin 1, which is also released by human epidermal cells, stimulates the production of GM-CSF in fibroblasts [3] and endothelial cells [4]. Thus, GM-CSF could play an important role in propagation of the inflammatory reaction in skin diseases. In addition, TNF, which shares a number of biologic characteristics with interleukin 1, was shown to stimulate various cell types (e.g., vascular endothelial cells, cultured normal lung fibroblasts, and smooth muscle cells) to produce GM-CSF [5,37,38] and to induce CSF production in vivo [39]. However, GM-CSF induces expression of the TNF gene by human monocytes and enhances TNF secretion by endotoxin-stimulated monocytes [40]. It is, therefore, considered that distinct biologic effects of GM-CSF may be amplified through the release of TNF which also represents an important granulocyte-activating cytokine [41,42].

Human GM-CSF may play a crucial role in activation of PMN in the skin, turning them into active scavengers of parasites and microbial organisms. However, if present in high concentrations at inflammatory sites, GM-CSF-induced granulocyte activation may be deleterious and relevant in the pathogenesis of inflammatory skin diseases.

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