Purification and Properties of Bacterially Synthesized Human Granulocyte-Macrophage Colony Stimulating Factor


Human granulocyte-macrophage colony stimulating factor (GM-CSF) has been synthesized in high yield using a temperature inducible plasmid in *Escherichia coli*. The human GM-CSF is readily isolated from the bacterial proteins because of its differential solubility and chromatographic properties. The bacterially synthesized form of the human GM-CSF contains an extra methionine residue at position 1, but otherwise it is identical to the polypeptide predicted from the cDNA sequence. The specific activity of 2.9 x 10^7 units/mg of protein for purified bacterially synthesized human GM-CSF indicates that despite the lack of glycosylation, the molecule is substantially in its native conformation. This molecule stimulated the same number and type of both seven- and 14-day human bone marrow colonies as the CSFa preparation from human placental conditioned medium. Human GM-CSF had no activity on murine bone marrow or murine leukemic cells. There was no detectable, direct stimulation of adult human erythroid burst forming units (BFU-E) by the bacterially synthesized human GM-CSF. Although impure preparations containing native human GM-CSF (eg, human placental conditioned medium) stimulated the formation of mixed colonies, even in the presence of erythropoietin, the bacterially synthesized human GM-CSF failed to stimulate the formation of mixed colonies from adult human bone marrow cells. The bacterially synthesized human GM-CSF increased N-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced superoxide production and lysozyme secretion. Antibody-dependent cytotoxicity and phagocytosis by human neutrophils was stimulated by the bacterially synthesized human GM-CSF and eosinophils were also activated in the antibody-dependent cytotoxicity assay.

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THE IDENTIFICATION of the cDNA clone for murine granulocyte-macrophage colony stimulating factor (mGM-CSF) enabled the production of the biologically active polypeptide by COS cells and bacteria. The cDNA mGM-CSF also led to the isolation of full-length cDNAs for human GM-CSF (hGM-CSF) and confirmed the identity of the human CSF cloned from a COS cell expression library. The hGM-CSF expressed by COS cells appears to encode all of the in vitro biological activities associated with CSFa prepared from human placental conditioned medium and conditioned medium from the U5637 cell line. The COS cell-derived hGM-CSF stimulated the proliferation and differentiation of granulocyte, macrophage, and eosinophil progenitor cells as well as the function of neutrophils. Thus, the simian-derived carbohydrate moiety does not appear to alter the cellular specificity of the hGM-CSF. However, it is possible that a hGM-CSF polypeptide with a simian, hamster, or yeast carbohydrate moiety will be antigenic in vivo or that the different carbohydrate moiety will influence the in vivo distribution of the GM-CSF. We have produced hGM-CSF in bacteria and purified the polypeptide without any attached carbohydrate. These studies define the conditions necessary for the preparation and purification of bacterially synthesized hGM-CSF, the specific activity of the molecule, and its activities on progenitor as well as mature cells. In part, these results emphasize the continuing need to understand the interactions between subsets of human bone marrow cells.

**MATERIALS AND METHODS**

*Preparation and Purification of Bacterially Synthesized Human GM-CSF*

hGM-CSF cDNA was isolated from a library constructed using mRNA from the human monocytic cell line U937 by screening candidate clones with a nick-translated probe derived from the coding sequences of mGM-CSF. The deduced amino acid sequence of the U937 hGM-CSF was identical to hGM-CSF produced by the leukemic cell line Mo(6) with the exception of the conservative substitutions of a methionine by an isoleucine (amino acid 80 of the mature protein) and a threonine by an isoleucine (amino acid 100 of the mature protein). The amino acid for residue 100 of the mature protein deduced from a genomic clone was also isoleucine. These differences between cDNAs and the genomic sequences may represent allelic variations. Sequences coding for the mature hGM-CSF protein were placed in an *Escherichia coli* vector behind an inducible promoter as previously described for the mGM-CSF gene. The construction took advantage of the unique restriction endonuclease site (Hgo I) located 58 nucleotides before the codon for the first amino acid of the mature protein (alanine). An oligonucleotide linker was synthesized to replace the deleted codons with the addition of an amino-terminal methionine codon to initiate translation in the prokaryote host. This linker contained substitutions of adenosine wherever retention of the native amino acid allowed. The noncoding 3′ distal end of the cDNA was cleaved at the unique Bal I restriction endonuclease site and ligated to a similarly blunt-ended site (Smal I) in the expression vector. Once introduced into a suitable *E. coli* host (LON-, HPTR-) the induction of hGM-CSF production was initiated by temperature shift (28°C to 48°C). hGM-CSF accumulated over four hours to 8% to 10% of the total cell protein. Bacteria were harvested and GM-CSF extracted essentially as described for mGM-CSF. In induced cells hGM-CSF accumulated from the Melbourne Tumour Biology Branch, Ludwig Institute for Cancer Research, Australia; the Walter and Eliza Hall Institute, Australia and Biogen SA Pty Ltd, Geneva.

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in dense bodies which were collected by centrifugation following
breakage of the cells. After washing the pellet was resuspended in 6
mol/L guanidinium hydrochloride and 2 mmol/L 1,6-mercaptoetha-
nol and chromatographed over G-100 Sephadex in the same buffer.
The highly purified hGM-CSF fractions were collected and the
denaturant and reductant removed by slow dialysis against neutral
Tris buffer. The final purification step was gradient elution from a
reverse-phase support at pH 2.1 on an Ultrapure RPSC column
(4.6 x 7.5 cm) from Beckman, Santa Clara, CA.17 The final purity
of the hGM-CSF was assessed by sodium dodecyl sulfate (SDS) gel
electrophoresis18 and automated gas phase amino acid sequence
analysis.19 The protein concentration for the specific activity calcu-
lated was determined by amino acid analysis.20

Assays on Bacterially Synthesized Human GM-CSF

The purified hGM-CSF preparation (approximately 0.3 mg/mL
in acetonitrile [30%]; trifluoroacetic acid [0.1%]; water [70%]) was
diluted 1:100 in 10% normal saline containing fetal calf serum
(FCS) (10%, vol/vol) and 0.1 mL of the diluted sample (or serial
dilutions thereof) was assayed in 1-mL semisolid bone marrow
cultures.

Human marrow cultures. Marrow and peripheral blood samples
were obtained with the prior informed consent of patients
according to a protocol approved by the Ethics Committee of the
Royal Melbourne Hospital. Light density (p 1.070 g/cc) or nonad-
ergent light density bone marrow cells (30,000) from patients
without a history of hematological disease were cultured in 35-mm Petri dishes
using 1 mL of Dulbecco’s Modified Eagle’s Medium and a final concentration
of 20% FCS and 0.3% agar.21 Colonies were incubated for up to
14 days at 37 °C in a fully humidified atmosphere of 10% CO2 in air.
For erythroid colony formation, 1-mL cultures containing 30,000
density (p<1.070 g/cc) mononuclear or nonadherent mononu-
clear bone marrow cells in Iscove’s Modified Dulbecco’s Medium
and a final concentration of 25% FCS and 0.3% agar were estab-
lished. Cultures were incubated for 14 days in a fully humidified
atmosphere of 5% CO2 in air.

Colonies formation (clones of more than 40 cells) was scored at 35 x
magnifications using a dissecting microscope after seven or 14 days
of incubation. To determine the cellular composition of the resulting
colonies, intact cultures were fixed using 2.5% glutaraldehyde and
methanol and, after drying, were stained with Luxol Fast-Blue/
haematoxycin. Colony classification was performed at
magnifications using a dissection microscope after seven or 14 days
of incubation. To determine whether bacterially synthesized hGM-CSF
was able to induce the differentiation of WEHI-3B(D+) cells,22 serial
dilutions of the hGM-CSF samples (0.1 mL) were added to agar
cultures of 300 WEHI-3B(D+) cells. The percentage of colonies
exhibiting differentiation was determined after seven days of incuba-
tion.23 Control cultures contained 0.1 mL of serial dilutions of either
serum from C57BL mice injected three hours previously with 5 µg
endotoxin24 or semipurified human CSF.25

Functional Activation of Mature Cells

Purification of human neutrophils and eosinophils. These cells
were obtained from the peripheral blood of healthy volunteers after
dextran sedimentation and centrifugation on a hypertonie gradient
of Metrizamide (Nyegaard, Oslo) as previously described.26 The
purity was higher than 95% for neutrophils and higher than 92% for
eosinophils. The cells were resuspended in Eagle’s Minimal Essential
Medium supplemented with 10% FCS, 20 mmol/L HEPES buffer,
and antibiotics.

Antibody-dependent cell mediated cytotoxicity assay. ADCC
assay was performed as previously described.23 In brief, 40 µL of 51Cr-
labeled, trinitrophenyl (TNP)-coupled P815 cells (4 x 105) were
incubated with 24 µL of rabbit IgG anti-TNP (Rehovot, Israel), 80
µL of purified human neutrophils or eosinophils (1.3 x 105) as
effectors cells, and 16 µL of bacterially synthesized hGM-CSF for
two and one half hours at 37 °C in V-bottom microtitre plates.
Percent cytotoxicity was calculated from the formula: (test –
control)/total x 100, where “control” was the 51Cr
released by P815 cells in the presence of medium alone, and “total” was the 51Cr
released by the addition of 5% Triton X-100.

Superoxide production. Purified neutrophils were incubated with
medium or different concentrations of bacterially synthesized
hGM-CSF for two hours at 37 °C. After this, 150 µL of cells (105) were mixed with 100 µL freshly prepared cytochrome-C (Sigma,
St Louis, type VI, 12.4 mg/mL), 100 µL N-formyl-methionyl-leucyl-
phenylalanine (FMLP) (10-4 mol/L) and made up to 1 mL with
medium. The mixtures were then incubated at 37 °C for five
minutes, after which the cells were rapidly cooled, centrifuged at
4 °C, and the supernatants transferred to plastic disposable cuvettes.
Superoxide production was measured by the reduction of cyto-
chrome-C27 using an extinction coefficient of 21.1 mmol/L.1.14

The medium used throughout these assays was RPMI (free of phenol
red) containing 2% FCS. In control experiments, bacterially
synthesized hGM-CSF did not reduce cytochrome-C in the absence
of cells, and superoxide dismutase (Sigma) completely abolished
the reduction of cytochrome-C. The results are expressed as the means
of triplicate determinations ± 1 standard deviation.

Phagocytosis assay. This assay measured the phagocytosis of
serum-opsonized baker’s yeast and was performed as described.8 In
brief, 100 µL purified neutrophils (2 x 107/mL) were incubated
with 250 µL baker’s yeast, 100 µL diluted fresh autologous serum
and 50 µL bacterially synthesized hGM-CSF or medium with FCS
for one hour at 37 °C. After this, the cells were centrifuged at 4 °C,
resuspended in 50 µL of cold PBS and smeared onto a slide. When
eosinophils were tested, this assay was scaled down because of the
relatively low numbers of eosinophils routinely obtained. The vol-

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BACTERIALLY SYNTHESIZED hGM-CSF

Volumes were 20 μL of cells (10^7/mL), 50 μL of baker’s yeast, 20 μL of freshly obtained human serum and 10 μL of bacterially synthesized hGM-CSF or medium. After incubation, cytocentrifuge preparations were made. Slides were fixed in methanol and stained with Giemsa before being examined for the number of cells showing different numbers of phagocytosed yeast. The data are expressed as percentages after counting a minimum of 200 cells per slide.

Degranulation assay. This was carried out as previously described. Briefly, purified neutrophils (5 x 10^6/mL) pretreated with cytochalasin-B (Sigma) were incubated with different stimuli for 15 minutes at 37 °C. To these mixtures different concentrations of FMLP (Sigma) were added and the cells incubated for another 30 minutes at 37 °C. After incubation the cells were centrifuged and the released lysozyme assayed against Micrococcus lysodeikticus (Sigma). The optical density at 450 nm was measured at ten-second intervals for three minutes. Triplicate determinations (every ten seconds) were made and the amount of lysozyme released was calculated from a standard curve generated by egg white lysozyme (Sigma). Maximum values were obtained by using supernatants from neutrophils lysed with Triton X-100.

RESULTS AND DISCUSSION

Induction and Purification of Bacterially Synthesized Human GM-CSF

Recombinant hGM-CSF was produced in E. coli under the regulation of a heat-inducible promoter. After GM-CSF synthesis was induced by raising the culture temperature, the protein accumulated in aggregates as dense bodies within the cells. Following weakening of the cell walls by lysozyme treatment, cells were disrupted in a French pressure cell. The aggregates were collected by centrifugation and resuspended in 6 mol/L urea. hGM-CSF was initially purified from the aggregates were collected by centrifugation and resuspended.

Fig 1. Reversed phase high-performance liquid chromatography purification of bacterially synthesized human GM-CSF.

Fig 2. Electrophoretic analysis of purified bacterially synthesized human GM-CSF.

yielded a single amino acid at each cycle; the first residue detected was methionine, and the next 16 cycles yielded the sequence expected from the nucleotide sequence. Thus, this product is different from the natural molecule in that not only is the carbohydrate moiety missing, but there is also an N-terminal methionine which is not removed during synthesis. The results of the quantitative amino acid analysis of the purified bacterially synthesized hGM-CSF were in excellent agreement with the composition predicted from the nucleotide sequence. The optical density at pH 2.1 in the presence of acetonitrile (30%, vol/vol), together with the amino acid analysis yielded a molar extinction coefficient of 2 x 10^4 moles^-1 cm^-1. Attempts to determine a precise specific activity for the purified bacterially synthesized hGM-CSF are complicated by the wide variation in sensitivity of different bone marrow samples and variations in the frequency of colony-forming cells for different human bone marrow samples. The standard assays were performed using between 30,000 and 50,000 bone marrow cells per milliliter. Eight separate bone marrows stimulated by the purified bacterially synthesized hGM-CSF yielded an average specific activity of 2.9 ± 1.9 x 10^3 U/mg of protein after seven days in culture. The same four samples yielded a specific activity of 2.9 ± 0.7 x 10^3 U/mg of protein after 14 days in culture. The ratio of day-7 to day-14 colonies was dependent on the bone marrow sample and varied from 0.3 to 1.6. In general, impure samples of hGM-CSF (eg, human placental conditioned medium or bladder carcinoma conditioned medium) contained a mixture of both hGM-CSF (CSFα) and hGM-CSF (CSFβ). The CSFβ preparation preferentially stimulates colony formation at day 7; thus, the ratio of the number of colonies at day 7 to day 14 (approximately three) tends to be considerably higher than for GM-CSF and the CSFα preparation. Similarly, a mixture of the bacterially synthesized GM-CSF and the CSFβ preparation generally stimulates more day-7 colonies than either stimulus by itself. Each molecule preferentially stimulates a defined subset of GM-progenitor cells. After 14 days in culture the bacterially
synthesized hGM-CSF stimulated as many colonies as preparations of CSFαβ from human placental conditioned media.11

Our method of preparation and purification indicates that the bacterially synthesized hGM-CSF is stable in 6 mol/L urea, as well as at pH 2.1 in the presence of 40% acetonitrile. Indeed, at low pH the bacterially synthesized hGM-CSF appears to retain its full biologic activity indefinitely. However, initial attempts to store this hGM-CSF at neutral pH in the absence of urea have led to precipitation of the molecule.

Concentration Dependence of Colony Formation

The purified hGM-CSF was titrated and assayed on eight separate bone marrow aspirates: both the seven-day (Fig 3A) and 14-day (Fig 3B) cultures indicated that maximal stimulation of colony formation was achieved at concentrations of 500 pmol/L or greater. Half-maximal stimulation occurred between 60 pmol/L and 120 pmol/L and the minimum detectable hGM-CSF concentration using the semisolid agar assay was 10 pmol/L for both time points.

The direct action of bacterially synthesized hGM-CSF on human colony forming cells was examined by transfer of day-5 clones to plates containing the bacterially synthesized hGM-CSF, human placental conditioned medium, or saline (Fig 4). The bacterially synthesized hGM-CSF continued to stimulate colony growth and GM-CSF is undoubtedly one of the active hematopoietic growth factors in the HPCM.

Morphology of Colonies

In two separate experiments, the types of colonies stimulated by the bacterially synthesized hGM-CSF have been compared to the colony types obtained with unfractionated HPCM, CSFα, and CSFβ (Table 1). The bacterially synthesized hGM-CSF stimulated pure neutrophil (G), macrophage (M) and eosinophil (Eo) colonies as well as mixed granulocyte-macrophage colonies. At day 7 all preparations of CSF stimulated mainly granulocytic colonies. However, the bacterially synthesized hGM-CSF and CSFα also stimulated a small proportion of Eo colonies. As expected, for both HPCM and CSFα the proportions of pure M and pure Eo colonies were increased at 14 days. Thus, the bacterially synthesized hGM-CSF stimulated a similar range of colonies to the CSFα.

The bacterially synthesized hGM-CSF was used in an attempt to stimulate the hematopoietic progenitor cells in murine bone marrow. Even at a concentration of 2 nmol/L no colonies or clusters were observed after seven days in culture. The hGM-CSF did not enhance the survival of multi-CSF-responsive progenitor cells in the delayed addition assay. Similarly, there was no effect of this hGM-CSF on several murine cell lines which were known to respond to murine hematopoietic growth factors: WEHI-3BD (G-CSF-responsive) did not differentiate in response to hGM-CSF, FDCP1 (GM-CSF- or Multi-CSF-dependent) and 32D cells (Multi-CSF-dependent) did not proliferate in the presence of hGM-CSF (data not shown).

Effect of Bacterially Synthesized Human GM-CSF on Erythroid Progenitor Cells

The colony types stimulated by human tissue-derived conditioned media (eg, human placental conditioned medium, [HPCM]) have been studied extensively using a range of partially purified hematopoietic progenitor cells.36,37 Studies by Sieff et al with a hGM-CSF partially purified from transfected COS cell conditioned medium indicated
Table 1. Morphology of Human Bone Marrow Colonies Stimulated by Bacterially Synthesized hGM-CSF

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Experiment Number</th>
<th>Stimulus</th>
<th>Mean Number of Colonies*</th>
<th>Colony Type (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>G</td>
<td>GM</td>
</tr>
<tr>
<td>7 days</td>
<td>1</td>
<td>Saline</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPCM</td>
<td>275</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hGM-CSF (recombinant)</td>
<td>230</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CSFα</td>
<td>232</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CSFβ</td>
<td>278</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Saline</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPCM</td>
<td>146</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hGM-CSF (recombinant)</td>
<td>98</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CSFα</td>
<td>98</td>
<td>56</td>
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<tr>
<td></td>
<td></td>
<td>CSFβ</td>
<td>149</td>
<td>64</td>
</tr>
<tr>
<td>14 days</td>
<td>1</td>
<td>Saline</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPCM</td>
<td>98</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hGM-CSF (recombinant)</td>
<td>95</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CSFα</td>
<td>93</td>
<td>52</td>
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<td></td>
<td></td>
<td>CSFβ</td>
<td>31</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Saline</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPCM</td>
<td>87</td>
<td>31</td>
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<td></td>
<td></td>
<td>hGM-CSF (recombinant)</td>
<td>89</td>
<td>23</td>
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<tr>
<td></td>
<td></td>
<td>CSFα</td>
<td>85</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CSFβ</td>
<td>30</td>
<td>67</td>
</tr>
</tbody>
</table>

*3 x 10⁴ light density nonadherent human bone marrow cells were cultured in 1 mL of semisolid agar and examined after seven and 14 days. The agar cultures were fixed and stained and the colonies analyzed to determine the proportion of G, neutrophil; M, macrophage; GM, neutrophil-macrophage and EOS, eosinophil colonies.

Table 2. No Effect of Bacterially Synthesized hGM-CSF on Erythroid Colony Formation From Mononuclear Human Bone Marrow Cells

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Day of Epo† Addition</th>
<th>Pure Erythroid</th>
<th>Mixed Erythroid</th>
<th>Non-erythroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>—</td>
<td>0</td>
<td>24 ± 5</td>
<td>0</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>HPCM</td>
<td></td>
<td>28 ± 2</td>
<td>98 ± 5</td>
<td></td>
</tr>
<tr>
<td>HPCM</td>
<td></td>
<td>0</td>
<td>0</td>
<td>110 ± 3</td>
</tr>
<tr>
<td>hGM-CSF (2.5 nmol/L)</td>
<td>—</td>
<td>0</td>
<td>11 ± 12</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (2.5 nmol/L)</td>
<td>0</td>
<td>20 ± 8</td>
<td>102 ± 4</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (50 nmol/L)</td>
<td>—</td>
<td>0</td>
<td>124 ± 11</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (50 nmol/L)</td>
<td>0</td>
<td>13 ± 5</td>
<td>104 ± 11</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>3</td>
<td>17 ± 6</td>
<td>16 ± 5</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>4</td>
<td>13 ± 5</td>
<td>17 ± 4</td>
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<tr>
<td>HPCM</td>
<td></td>
<td>16 ± 1</td>
<td>117 ± 5</td>
<td></td>
</tr>
<tr>
<td>HPCM</td>
<td></td>
<td>15 ± 6</td>
<td>121 ± 13</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (2.5 nmol/L)</td>
<td>—</td>
<td>21 ± 8</td>
<td>87 ± 9</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (2.5 nmol/L)</td>
<td>4</td>
<td>21 ± 4</td>
<td>65 ± 10</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (50 nmol/L)</td>
<td>3</td>
<td>14 ± 2</td>
<td>99 ± 9</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (50 nmol/L)</td>
<td>4</td>
<td>12 ± 1</td>
<td>104 ± 15</td>
<td></td>
</tr>
</tbody>
</table>

—, Epo not added.
†Partially purified erythropoietin (Epo) was added to a final concentration of 1.5 U/mL either at the start of the cultures (day 0) or at three or four days after initiating the assay.
‡For all assays three replicate plates were scored after 14 days. The table entries represent the mean ± SD.
Table 3. No Effect of Bacterially Synthesized hGM-CSF on Erythroid Colony Formation from Nonadherent Mononuclear Human Bone Marrow Cells

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Day of Epo Addition</th>
<th>Pure Erythroid</th>
<th>Mixed Erythroid</th>
<th>Non-erythroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPCM</td>
<td>0</td>
<td>0</td>
<td>14 ± 2</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (2.5 nmol/L)</td>
<td>0</td>
<td>0</td>
<td>13 ± 3</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (25 nmol/L)</td>
<td>0</td>
<td>22 ± 1</td>
<td>97 ± 1</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (50 nmol/L)</td>
<td>0</td>
<td>26 ± 1</td>
<td>217 ± 5</td>
<td></td>
</tr>
<tr>
<td>HPCM</td>
<td>0</td>
<td>31 ± 7</td>
<td>95 ± 2</td>
<td></td>
</tr>
<tr>
<td>HPCM</td>
<td>4</td>
<td>31 ± 5</td>
<td>90 ± 3</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (2.5 nmol/L)</td>
<td>3</td>
<td>11 ± 2</td>
<td>104 ± 6</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (25 nmol/L)</td>
<td>4</td>
<td>9 ± 2</td>
<td>105 ± 6</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (50 nmol/L)</td>
<td>3</td>
<td>16 ± 1</td>
<td>191 ± 2</td>
<td></td>
</tr>
<tr>
<td>hGM-CSF (50 nmol/L)</td>
<td>4</td>
<td>4 ± 1</td>
<td>206 ± 11</td>
<td></td>
</tr>
</tbody>
</table>

*See footnote to Table 2 for technical details of these experiments.

After removing the adherent cells, HPCM but not the bacterially synthesized hGM-CSF increased the cloning efficiency of the erythroid progenitor cells (Table 3). When the adherent cells are removed, a subset of the erythroid progenitors appears to be stimulated by HPCM. Even when the erythropoietin is added three or four days after the initiation of the cultures with HPCM this synergistic effect of HPCM can be observed (Table 3). Again, approximately half the erythroid colonies appear to be stimulated by a molecule present in human placental conditioned medium. It is unlikely that GM-CSF is related to this phenomenon, as the bacterially synthesized hGM-CSF did not modulate the number of 14-day erythroid colonies.

When both erythropoietin and hGM-CSF were present, their effects appeared to be independent. No extra erythroid colonies were observed compared to the cultures stimulated with erythropoietin alone. In contrast, unfractionated HPCM consistently increased (two- to fourfold) the number of erythroid colonies developing from the nonadherent light density mononuclear bone marrow cells. Mixed erythroid colonies were only observed in the presence of the unfractionated HPCM. Neither the bacterially synthesized GM-CSF, the erythropoietin, nor the mixture of both were able to stimulate human mixed colonies. At concentrations greater than 500 pmol/L, the bacterially synthesized GM-CSF stimulated the same range of nonerythroid colonies as the optimum concentration of human placental conditioned medium.

The lack of erythroid (BFU-E) potentiation by hGM-CSF could be due to the presence of accessory cells which suppress the GM-CSF-responsive BFU-E. However, the previously reported effects of GM-CSF may also be indirect (ie, via a GM-CSF-responsive cell which produces an erythropoietin factor). Understanding the interactions between the different cells controlling human erythropoiesis will require a detailed analysis of the proliferation of single cells and subsequent cell-mixing experiments.

Action of Bacterially Synthesized hGM-CSF on Mature Hematopoietic Cells

As well as stimulating the proliferation of the myeloid progenitor cells, the hGM-CSF purified from the Mo cell line is able to stimulate some of the functions of mature neutrophils and eosinophils. We have measured the effect of the bacterially synthesized GM-CSF on four parameters of granulocyte function: superoxide (O2· −) production, phagocytosis, lysozyme secretion, and antibody-dependent cell-mediated cytotoxicity.23 At concentrations of hGM-CSF above 10 pmol/L both purified neutrophils and eosinophils were stimulated maximally to kill tumor target cells, and stimulation of killing was still detectable at 1 pmol/L (Fig 5). The concentration dependence of neutrophil and eosinophil activation was very similar (Fig 5). A similar concentration-dependent effect on neutrophil function was obtained by Weisbart et al for the inhibition of neutrophil migration using either recombinant hGM-CSF produced by transfected monkey COS cells or the hGM-CSF purified from the Mo cell line. Thus, our results show that hGM-CSF does not require carbohydrate moieties for its action in vitro.

COS cell–derived hGM-CSF has been previously found to stimulate phagocytosis.24 A comparison of the effects of E. coli–synthesized hGM-CSF on neutrophil phagocytosis of yeast organisms in the presence or absence of serum is displayed in Fig 6. In the presence of fresh human serum, the bacterially synthesized hGM-CSF (4 nmol/L) activated large numbers of neutrophils to phagocytose several yeast particles (Fig 6). Heating of human serum for 30 minutes at 56 °C abrogated the GM-CSF stimulation of neutrophils to phagocytose heat-killed yeast (data not shown), suggesting that complement is the opsonic factor present in human serum, and that the stimulation by hGM-CSF is dependent on the presence of this opsonin. There was a 50% increase in the number of neutrophils which phagocytosed the heat-killed yeast (Fig 6).

The response of neutrophils to bacterial products also appears to be stimulated by hGM-CSF. The secretion of lysozyme by neutrophils was dependent on the presence of chemotactic agents (Table 4). In the absence of a chemotactic peptide such as FMLP,39 there was no activation of lysozyme secretion. However, preincubation of neutrophils with the bacterially synthesized hGM-CSF enhanced the FMLP-induced release of lysozyme from purified neutrophils by 60% (Table 4). At low concentrations of the chemotactic peptide (10−8 mol/L) the hGM-CSF was more...
BACTERIALLY SYNTHESIZED hGM-CSF

Fig 6. Effect of GM-CSF (4 nmol/L) on the ability of human neutrophils to phagocytose yeast.

effective than lipopolysaccharide.43 Bacterially synthesized hGM-CSF also enhanced FMLP-mediated stimulation of superoxide production by human neutrophils (Fig 7). In the absence of hGM-CSF and FMLP, superoxide production by human neutrophils was not detectable. The chemotactic peptide by itself induced O$_2^-$ production to 1.3 nnoles/min/10^6 cells. In the absence of FMLP, hGM-CSF had little or no effect on O$_2^-$ production (Fig 7). However, preincu- 
bation of neutrophils with GM-CSF followed by FMLP increased the amount of O$_2^-$ production sevenfold over the level induced by FMLP alone. The interaction between FMLP and GM-CSF was concentration dependent: the maximal rate of O$_2^-$ production was achieved at 200 pmol/L, but the synergism was still evident when the hGM-CSF concentration was 10 pmol/L (Fig 7).

Thus, GM-CSF is capable of enhancing at least three separate systems in human neutrophils: the release of both lysozyme and O$_2^-$ (upon stimulation with FMLP), phagocytosis of yeast (complement-mediated), and killing of tumor target cells (antibody-dependent).44 In its ability to activate mature neutrophils, this bacterially-synthesized hGM-CSF behaves very similarly to the recombinant hGM-CSF produced by transfected monkey COS cells (ref 9, and Lopez et al, in preparation).

We have demonstrated the action of purified, bacterially synthesized hGM-CSF on a range of hematopoietic progenitor and mature cells. This GM-CSF has a specific activity in the same range as the recombinant GM-CSF purified from medium conditioned by Mo cells45 or the medium from COS cells transfected with cDNA for hGM-CSF12 (Table 5). Thus, neither the addition of the extra N-terminal methionine residue, nor the absence of carbohydrate appear to affect the in vitro activity of hGM-CSF. The detailed morphological distribution of the colonies stimulated by both the E. coli-synthesized and COS cell–stimulated hGM-CSF varies considerably and is dependent on the specific bone marrow being assayed. However, both forms stimulate predominantly neutrophilic granulocyte colonies at day 7 (Table 5), and by day 14 most of the colonies contain macrophages.

<table>
<thead>
<tr>
<th>Table 4. Effect of Bacterially Synthesized hGM-CSF on Lysozyme Release by Neutrophils</th>
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<tbody>
<tr>
<td>Degranulating Lysozyme Secretion* Following Preincubation</td>
</tr>
<tr>
<td>with hGM-CSF (pmol/L)†</td>
</tr>
<tr>
<td>Nil</td>
</tr>
<tr>
<td>FMLP 10^{-7} mol/L</td>
</tr>
<tr>
<td>FMLP 10^{-8} mol/L</td>
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*Lysozyme secretion expressed as the average U/mL/min/10^6 neutrophils ± SD (for three replicate cultures).
†Final concentration of bacterially synthesized hGM-CSF.

Table 5. Comparison of E. coli-Derived and COS Cell–Derived hGM-CSF

<table>
<thead>
<tr>
<th>Property</th>
<th>E. coli-Derived</th>
<th>COS Cell-Derived</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent molecular weights</td>
<td>16,000</td>
<td>19,000–22,000</td>
</tr>
<tr>
<td>Specific Activity (U/mg/10^6 bone marrow cells)</td>
<td>1 x 10^7</td>
<td>1 x 10^2–5 x 10^7</td>
</tr>
<tr>
<td>Morphology of myeloid colonies (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>Day 14</td>
<td>Day 8</td>
</tr>
<tr>
<td>G</td>
<td>70</td>
<td>31</td>
</tr>
<tr>
<td>GM</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>M</td>
<td>24</td>
<td>47</td>
</tr>
<tr>
<td>Eo</td>
<td>2</td>
<td>14</td>
</tr>
</tbody>
</table>

BFU-E Responsiveness: No effect
(a) Stimulated
(b) No effect
(c) Inhibitory in the presence of erythroid potentiating activity

Responsive mature cells: G, Eo, GM, B1, macrophages
Abbreviations: G, granulocytes; GM, granulocyte–macrophages; M, monocytes; Eo, eosinophils.
nant hGM-CSF also reported that there was no effect on BFU-E, although in the presence of erythropoietin and erythropoietin, the human GM-CSF appeared to inhibit BFU-E. Others appear to have shown effects of COS cell–derived hGM-CSF on highly purified BFU-E. It will be necessary to study the effects of pure hGM-CSF on single BFU-E before we can be sure that the hGM-CSF has a direct effect on erythropoiesis.

The availability of *E. coli*–synthesized hGM-CSF with essentially identical biologic properties to natural hGM-CSF will provide sufficient quantities of this molecule for clinical testing. The absence of any foreign carbohydrate moiety should reduce problems with immunologic neutralization during clinical applications. The absence of carbohydrate side chains has allowed the development of monoclonal antibody reagents which can distinguish between the native hGM-CSF (as is present in human placental conditioned medium) and the bacterially synthesized GM-CSF. Thus, it will be possible to monitor the serum levels of the administered GM-CSF during patient treatment. The bacterially synthesized hGM-CSF should be suitable both for studies aimed at increasing the rate of recovery of myeloid cells after cytotoxic treatment and for attempts to activate neutrophils and/or eosinophils in patients with poor neutrophil function, eg, older patients, immunosuppressed patients or patients with chronic infections.

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Purification and properties of bacterially synthesized human granulocyte-macrophage colony stimulating factor

AW Burgess, CG Begley, GR Johnson, AF Lopez, DJ Williamson, JJ Mermod, RJ Simpson, A Schmitz and JF DeLamarter

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