Recombinant Human Granulocyte Colony-Stimulating Factor: Effects on Normal and Leukemic Myeloid Cells

LAWRENCE M. SOUZA, THOMAS C. BOONE, JANICE GABRILOVE, POR H. LAI, KRISZTINA M. ZSEBO, DOUGLAS C. MURDOCK, VICKI R. CHAZIN, JOAN BRUSZEWSKI, HSIENG LU, KENNETH K. CHEN, JEAN BARENDT, ERICH PLATZER, MALCOLM A. S. MOORE, ROLAND MERTELSMANN, KARL WELTE

Experiments were conducted to isolate and characterize the gene and gene product of a human hematopoietic colony-stimulating factor with pluripotent biological activities. This factor has the ability to induce differentiation of a murine myelomonocytic leukemia cell line WEHI-3B(D^+) and cells from patients with newly diagnosed acute nonlymphocytic leukemia (ANLL). A complementary DNA copy of the gene encoding a pluripotent human granulocyte colony-stimulating factor (hG-CSF) was cloned and expressed in *Escherichia coli*. The recombinant form of hG-CSF is capable of supporting neutrophil proliferation in a CFU-GM assay. In addition, recombinant hG-CSF can support early erythroid colonies and mixed colony formation. Competitive binding studies done with ^{125}I-labeled hG-CSF and cell samples from two patients with newly diagnosed human leukemias as well as WEHI-3B(D^+) cells showed that one of the human leukemias (ANLL, classified as M4) and the WEHI-3B(D^+) cells have receptors for hG-CSF. Furthermore, the murine WEHI-3B(D^+) cells and human leukemic cells classified as M2, M3, and M4 were induced by recombinant hG-CSF to undergo terminal differentiation to macrophages and granulocytes. The secreted form of the protein produced by the bladder carcinoma cell line 5637 was found to be *O*-glycosylated and to have a molecular weight of 19,600.

THE PROLIFERATION AND DIFFERENTIATION of hematopoietic cells is under the control of specific growth stimuli known as colony-stimulating factors (CSF's) (*1*). The biological effects of these molecules have been demonstrated in semisolid culture systems that allow proliferation and differentiation of hematopoietic progenitor cells (*2, 3*).

Four murine growth regulatory glycoproteins have been identified. Murine granulocyte-macrophage (GM-CSF) and macrophage (M-CSF) colony-stimulating factor, which support the growth of granulocyte-macrophage and macrophage committed progenitors, respectively, have been highly purified (*4, 5*). Murine interleukin-3 (IL-3), a multilineage hematopoietic growth factor that supports the growth of erythroid cells, megakaryocytes, neutrophils, macrophages, and mast cells and possibly induces lymphoid proliferation and differentiation in vitro, has also been purified to apparent homogeneity (*6*). The fourth growth factor, termed granulocyte colony-stimulating factor (G-CSF), has been highly purified and is distinguished by its ability to stimulate neutrophilic granulocyte colonies in semisolid agar (*7*) and to induce the terminal differentiation and suppress the self-renewal capacity of murine myelomonocytic leukemic cells in vitro (*8, 9*).

Two of the murine CSF genes have been cloned, IL-3 (*10, 11*) and GM-CSF (*12*). The human equivalent of the murine GM-CSF gene has also been isolated (*13*), and most recently the human M-CSF analogous to murine M-CSF has been cloned (*14*). Human factors corresponding to murine IL-3 and G-CSF have not been cloned; however, a factor identified as human pluripotent CSF has properties that encompass the activities of murine IL-3 and G-CSF (*15, 16*). Human GM-CSF has also been shown to be a multilineage hemopoietin with many biological activities similar to murine IL-3 (*17*).

The human bladder carcinoma cell line 5637 constitutively produces colony-stimulating activities for committed myeloid as well as multipotential progenitors (*15, 16, 18–20*). One of the CSF's produced by 5637, pluripotent CSF, has been purified to near homogeneity (*15*). It supports the growth of human day-7 neutrophil colonies as well as early erythroid bursts (BFU-E) and mixed colony progenitors [colony-forming unit—granulocyte, erythroid, macrophage, megakaryocyte (GEMM)] from human bone marrow depleted of adherent cells and T lymphocytes (*15, 16*). In addition, it promotes the differentiation of the murine myelomonocytic WEHI-3B(D^+) leukemia cell line.

We report here the cloning of the gene for the factor known as pluripotent human CSF (*15*). Because of its similarities to murine G-CSF, we refer to this factor as human G-CSF (hG-CSF). The similarities to murine G-CSF include the ability of hG-CSF to generate predominantly granulocyte colonies in an assay for colony-forming units–granulocyte-macrophage (CFU-GM) (*16*) and induce terminal differentiation of WEHI-3B(D^+) cells (*15, 16*).

When hG-CSF was purified from the bladder carcinoma cell line 5637 (subclone 1A6) (*21*) and analyzed on a polyacrylamide gel stained with silver, it was greater than 95 percent pure (Fig. 1A, lane 2). Fourteen micrograms of highly purified hG-CSF was subjected to NH_2-terminal amino acid sequence analysis by means of a gas phase microsequencer. The phenylthiohydantoin-amino acids (PTH-aa) obtained for the first 40 cycles were identified by reversed-phase high-performance liquid chromatography (HPLC) (*22*) (Fig. 2b). The region containing amino acids 23–30 was used to deduce oligodeoxynucleotide probes of 23 bases in length. The oligonucleotides were complementary to the messenger RNA (mRNA) with the exception of inosine residues (*23*) opposite the third base of codons that were fourfold degenerate (positions 26, 28, and 29). The use of inosine allowed us to reduce the combination of probes from 1536 to 24. These probes were labeled with ^{32}P and hybridized to filters containing 150,000 complementary DNA (cDNA) clones constructed from 1A6 poly(A)$^+$ mRNA with the use of the Okayama and Berg vectors (*24*). Two positive clones were obtained by using the mixed probes. One clone, hG-CSF2, was 50 to 100 bp longer than the other at the 5′ end as determined by restriction endonuclease mapping. The entire insert in plasmid phG-CSF2 was sequenced by the dideoxy method (*25*) with the use of M13 templates containing various overlapping restriction endonuclease fragments (both orientations) of the hG-CSF gene (Fig. 2B). The deduced amino acid sequence (Fig. 2B) shows the cDNA insert encodes the entire mature form of hG-CSF as produced by 5637(1A6) cells. The insert, however, lacks a complete gene sequence, because there is no initiation codon (ATG) upstream of the first codon of the secreted form of hG-CSF (Fig. 2B). Comparing the deduced amino acid sequence of hG-CSF to those published for human M-CSF (*14*), human and mouse GM-CSF (*12, 13*), and mouse IL-3 (*10, 11*), we find no significant homology.

An expression vector was constructed to produce sufficient quantities of hG-CSF for

L. M. Souza, T. C. Boone, P. H. Lai, K. M. Zsebo, D. C. Murdock, V. R. Chazin, J. Bruszewski, H. Lu, K. K. Chen, Amgen, Thousand Oaks, CA 91320.
J. Gabrilove, M. A. S. Moore, R. Mertelsmann, K. Welte, Laboratory of Developmental Hematopoiesis and Laboratory of Molecular Hematology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.
J. Barendt, Amgen Development, Inc., Boulder, CO 80301.
E. Platzer, Institute for Clinical Immunology and Rheumatology, University of Erlangen, 8520 Erlangen, Federal Republic of Germany.

in vitro and in vivo biological studies. The vector was assembled via a three-way DNA ligation involving a modified *ts*-runaway plasmid (*26*) with a λP_L promoter, the hG-CSF gene, and a synthetic DNA fragment (44 bp) containing an initiation codon followed by the sequence encoding the mature form of hG-CSF (Fig. 3). *Escherichia coli* containing the p536hG-CSF2 expression plasmid were grown and induced to produce recombinant hG-CSF (rhG-CSF) (*27*). This rhG-CSF, when solubilized with a buffer containing sodium laurate and eluted from a C_4 HPLC column (*27*), is ≥95 percent pure (Fig. 1B, lane 1). Other purification procedures can be used to yield material with similar purity (Fig. 1A, lane 3).

As a measure of purity, the *E. coli*–derived rhG-CSF was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (5 percent 2-mercaptoethanol) (Fig. 1A, lane 3) or nonreducing conditions (Fig. 1A, lanes 5 and 6). The only bands detectable after silver staining were those of rhG-CSF. Whereas the 1A6 hG-CSF purified from tissue culture migrated with an apparent molecular weight (MW) of 19,600 (Fig. 1A, lane 2), the *E. coli* rhG-CSF had an apparent MW of 18,800 (Fig. 1A, lane 3). Both the native and recombinant hG-CSF's migrated with a slightly lower apparent molecular weight when loaded and run unreduced (Fig. 1A, lanes 4 to 6). Since pyrogens stimulate the endogenous release of CSF's from macrophages (*28*), we tested the highly purified rhG-CSF for endotoxin contamination using a limulus amebocyte lysate (LAL) assay (*29*). The purified rhG-CSF, at a concentration of 0.5 mg/ml, contained less than 0.5 ng of pyrogen per milliliter.

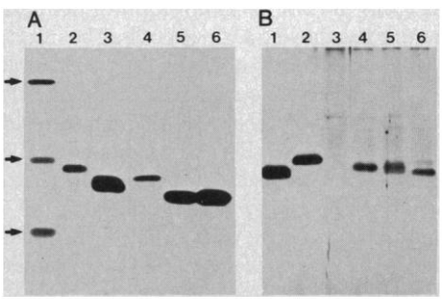

Fig. 1. Analysis of hG-CSF and rhG-CSF on a 15% (weight volume) SDS–polyacrylamide gel stained with silver. (A) Purified hG-CSF and rhG-CSF: lane 1, MW markers (30,000, 20,100, and 14,400); lane 2, 0.3 μg of hG-CSF reduced and boiled; lane 3, 1 μg of rhG-CSF reduced and boiled; lane 4, 0.3 μg of hG-CSF unreduced; lanes 5 and 6, 0.5 μg and 1 μg of rhG-CSF, respectively, unreduced. (B) Removal of *O*-glycans from hG-CSF: lane 1, rhG-CSF; lane 2, hG-CSF; lane 3, *O*-glycanase enzyme control; lane 4, hG-CSF treated with neuraminidase; lanes 5 and 6, hG-CSF treated with neuraminidase and then *O*-glycanase for 30 or 120 minutes, respectively.

It was possible that the difference in MW between the native hG-CSF and rhG-CSF was due to *O*-glycosylation, because (i) other known CSF's are glycosylated (*1*), (ii) the DNA sequence predicts the absence of asparagine residues which effectively rules out *N*-glycosylation, and (iii) the MW from the deduced amino acid sequence is 18,700, in close agreement with the MW measured for rhG-CSF, suggesting a post-translational modification of the native hG-CSF. To test for this possibility, we treated native hG-CSF with neuraminidase and then with *O*-glycanase (*30*) and then subjected it to SDS-PAGE. The MW of neuraminidase treated hG-CSF was measured at 19,200 (Fig. 1B, lane 4) and the subsequent treatment with *O*-glycanase reduced most of the hG-CSF to a MW of 18,800 (Fig. 1b, lanes 5 and 6). Since *O*-glycanase is a highly specific endoglycosidase (*31*), the reduction in MW of hG-CSF by neuraminidase and *O*-glycanase treatment suggests the following structure for the carbohydrate component: *N*-acetylneuraminic acid α(2-6)[galactose β(1-3)]*N*-acetylgalactosamine-R, where R is serine or threonine.

Pluripotent hG-CSF and rhG-CSF cause human bone marrow cells to proliferate and differentiate. These activities can be measured in CFU-GM (*32*), BFU-E, and CFU-GEMM assays (*33*) with the use of low density, nonadherent bone marrow cells from healthy human volunteers. A comparison of CFU-GM, BFU-E, and CFU-GEMM biological activities with 500 units of hG-CSF or rhG-CSF are shown in Table 1. Colonies formed in the CFU-GM assay were all positive for chloracetate esterase and negative for nonspecific esterase (α-naphthyl acetate esterase), consistent with the colonies being granulocytic in type. Both hG-CSF and rhG-CSF had a specific activity (*7*) of approximately 1×10^8 units per milligram of pure protein when assayed by serial dilution in a CFU-GM assay. The BFU-E and CFU-GEMM data in Table 1 are representative of three separate experiments and are similar to previous data for hG-CSF (*15*). The rhG-CSF is extremely pure and free of other potential mammalian growth factors by virtue of its production in *E. coli*. Thus rhG-CSF can support mixed colony formation and early erythroid bursts when added in the presence of recombinant erythropoietin. Until pure populations of hematopoietic progenitor cells become available, it will be difficult to determine whether hG-CSF acts directly to support the growth of mixed colonies or through a second signal provided by an as yet undetermined accessory cell. Therefore, it will be important to determine the nature and distribution of hG-CSF receptors among the various hematopoietic cell types.

The induction of differentiation of WEHI-3B(D^+) cells and leukemic cells by hG-CSF and rhG-CSF is shown in Fig. 4. The murine cell line WEHI-3B(D^+) undergoes differentiation to granulocytes and macrophages when exposed to murine G-CSF (*34*) or hG-CSF (*15*). This results in

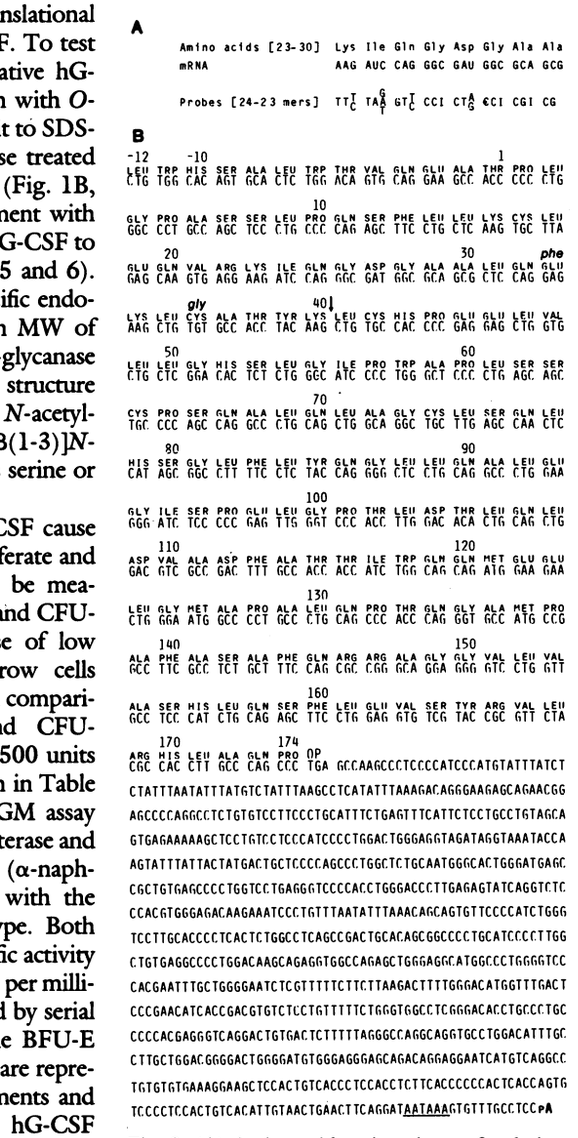

Fig. 2. (A) Amino acid region chosen for design of oligodeoxynucleotides complementary to hG-CSF mRNA. (B) DNA sequences of hG-CSF obtained from cDNA plasmid phG-CSF2 and the deduced amino acid sequence. The threonine was assigned amino acid position 1 on the basis of NH_2-terminal microsequencing of purified hG-CSF. The first 40 amino acids assigned by protein microsequencing are shown beginning at position 1 and ending at 40 (marked with an arrow). The two italicized amino acids above positions 33 and 36 depict the only amino acids incorrectly assigned by protein sequencing. The putative polyadenylation signal is shown underlined and the position at which poly(A) is added is depicted by pA.

suppression of proliferation and terminally differentiated progeny. Both hG-CSF and rhG-CSF cause 50 percent differentiation in a WEHI assay (*35*) with 50 to 100 units as defined in the CFU-GM assay.

It has been reported that WEHI-3B(D^+) cells and human cells from patients with newly diagnosed leukemias will bind ^{125}I-labeled murine G-CSF and that this binding can be competed for by addition of unlabeled G-CSF or human CSF-β (*19*). We tested the ability of hG-CSF and rhG-CSF to compete for binding of ^{125}I-hG-CSF to human and murine leukemic cells. Highly purified hG-CSF (>95 percent pure; 1 μg) was iodinated (*36*) and separated from reactants by gel filtration and ion exchange chromatography (*34*). The specific activity of the ^{125}I-hG-CSF was approximately 65 μCi per microgram of protein. Murine WEHI-3B(D^+) and two human peripheral blood myeloid leukemic cell preparations (ANLL, one classified as M4, the other as M5B) were tested for their ability to bind ^{125}I-hG-CSF (*37*). As shown in Table 2, ^{125}I-hG-CSF bound to the WEHI-3B(D^+) leukemic cells. The binding was inhibited in a dose-dependent manner by unlabeled hG-CSF or rhG-CSF, but not by GM-CSF. We also observed binding of hG-CSF to human myelomonocytic leukemic cells (ANLL and M4, Table 1). In liquid cultures these cells were induced by hG-CSF to differentiate into mature macrophages as judged by morphology. The absence of binding of ^{125}I-hG-CSF to monocytic leukemic cells from another patient (ANLL and M5B, Table 2) suggests that certain leukemias may differentially express or lack receptors for hG-CSF. The prognostic significance of this finding is unknown. The ability of rhG-CSF, like hG-CSF, to compete for the binding of ^{125}I-hG-CSF suggests that the receptors recognize both forms equally well. The role of glycosylation of hG-CSF, however, in the binding of hG-CSF to its receptor remains to be determined. Figure 4B shows that hG-CSF can induce granulocytic and monocytic differentiation of light-density bone marrow cells obtained from one patient with acute promyelocytic leukemia (M3) and another with acute myeloblastic leukemia (M2). Cells from each patient were cultured for 4 days in medium alone or in the presence of 1×10^5 units of rhG-CSF. Cells from the M3 control cultures incubated in medium alone (Fig. 4B, panel a) remained promyelocytic, whereas cells cultured in the presence of rhG-CSF showed mature cells of the myeloid type including a metamyelocyte, giant band forms and segmented neutrophils and monocytes (Fig. 4B, panel b). Whereas the control cells for this patient were 100 percent promyelocytes, the rhG-CSF treated cells included 22 percent blasts plus promyelocytes, 7 percent myelocytes, 35 percent metamyelocytes, 20 percent band forms plus segmented neutrophils, 14 percent monocytes, and 2 percent macrophages. One of the polymorphonuclear granulocytes shown in Fig. 4B, panel b (upper right-hand corner), still contains a prominent auer rod, suggesting that this cell represents a differentiated cell belonging to the leukemic clone. Cells from the second patient that were cultured in medium alone showed large "blast-like" cells (Fig. 4B, panel c). Some of the M2 cells, when treated with rhG-CSF, differentiated to mature segmented neutrophils (Fig. 4B, panel d). In Fig. 4B, panel d, there are residual auer rods in the center neutrophil suggesting the occurrence of differentiation in a cell belonging to the leukemic clone. Whereas the control M2 cells were 100 percent blasts, the rhG-CSF treated cells consisted of 43 percent blasts, 1 percent myelocytes, 15 percent metamyelocytes, 28 percent band forms plus segmented neutrophils, 2 percent promonocytes, and 11 percent monocytes. The leukemic cells were also examined for differentiation at four other concentrations of rhG-CSF (5×10^3, 1×10^4, 2.5×10^4, and 5×10^4 unit/ml). Even at 5×10^3 unit/ml there was significant differentiation (beyond myelocytes) of the M3 (50 percent) and M2 (37 percent) leukemic cells.

The ability to produce large quantities of hG-CSF and other human CSF's by means of recombinant technology has practical and theoretical implications. The multipotent activities of hG-CSF make it a good candidate for treatment of a number of hematopoietic disorders. Its ability to stimulate

Table 1. Comparison of CFU-GM, BFU-E, and CFU-GEMM activities in response to 500 units of hG-CSF or rhG-CSF. All the colony assays were performed with low-density nonadherent bone marrow cells. Human bone marrow cells were subjected to a density reduction with Ficoll-Hypaque (density, 1.077 g/cm^3; Pharmacia). The low-density cells were then resuspended in Iscove's modified Dulbecco's medium containing fetal calf serum (FCS) and placed for adherence on Falcon tissue culture dishes (No. 3003, Becton Dickenson) for 1 1/2 hours at 37°C.

CSF	CFU-GM*	BFU-E†	CFU-GEMM†
Medium‡	0 ± 0	26 ± 1	0 ± 0
hG-CSF	83 ± 5.4	83 ± 6.7	4 ± 0
rhG-CSF	87 ± 5.0	81 ± 0.1	6 ± 2

*For CFU-GM, target cells were plated at 1×10^5 in 1 ml of 0.3% agar that included supplemented McCoy's 5A medium and 10% heat inactivated FCS as described (*32*). Cultures were scored for colonies (greater than 40 cells per aggregate) and morphology was assessed on day 7 of culture. The number of colonies is shown as the mean ± SEM as determined from quadruplicate plates. †For BFU-E and CFU-GEMM, cells (1×10^5) were added to a 1-ml mixture of Iscove's modified Dulbecco's medium (Gibco), 0.8% methylcellulose, 30% FCS, 0.05 m*M* 2-mercaptoethanol, 0.2 m*M* hemin, and 1 unit of recombinant erythropoietin. Dishes were incubated in a humidified atmosphere of 5% CO_2 and 5% O_2. Low oxygen tension was obtained by means of an oxyreducer (Reming Bioinstruments). Colonies were scored after 14 days of incubation. The number of colonies is shown as the mean ± SEM, as determined from duplicate plates. ‡Medium control consisted of Iscove's modified Dulbecco's medium plus 10% FCS (CFU-GM) or 30% FCS, 0.2 m*M* hemin, and 1 unit of recombinant erythropoietin (CFU-GEMM and BFU-E).

Table 2. Specific binding of ^{125}I-hG-CSF to murine and human leukemic cells and inhibition of binding by hG-CSF and rhG-CSF. Specific binding (counts per minute) was determined as total binding in the absence of a competitor (mean of duplicates) minus binding (counts per minute) in the presence of 100-fold excess of unlabeled hG-CSF (nonspecific binding). The nonspecific binding was maximally 2503 cpm for WEHI-3B(D^+) cells, 1072 cpm for ANLL (M4) cells, and 1125 cpm for ANLL (M5B) cells. Experiments 1 and 2 were run on separate days with the same preparation of ^{125}I-hG-CSF and displayed internal consistency in the percentage inhibition noted for 2000 units of hG-CSF.

Competitor	Concentration (unit/ml)	WEHI-3B (D+)		ANLL (M4)		ANLL (M5B)	
		cpm	Inhibition (%)	cpm	Inhibition (%)	cpm	Inhibition (%)
			Experiment 1				
None	0	6,608		1,218		122	
hG-CSF	10,000	685	90				
	2,000	1,692	74	34	97	−376	0
	200	2,031	69				
rhG-CSF	10,000	0	100				
	2,000	1,185	82	202	83	0	0
	200	2,330	65				
			Experiment 2				
None	0	2,910	0				
hG-CSF	2,000	628	78				
GM-CSF	2,000	3,311	0				

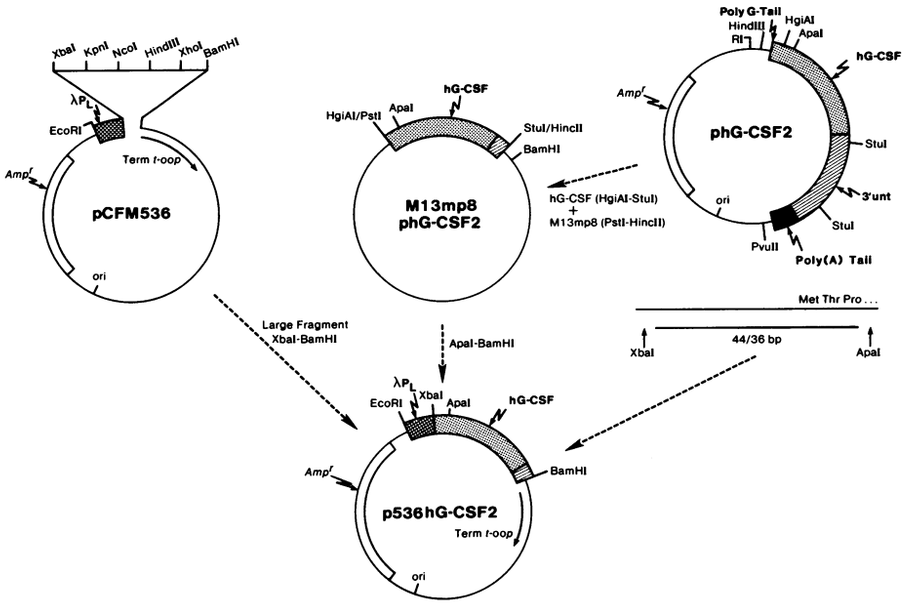

Fig. 3. Schematic of the constructions used to generate an *E. coli* expression vector for production of nonglycosylated phG-CSF.

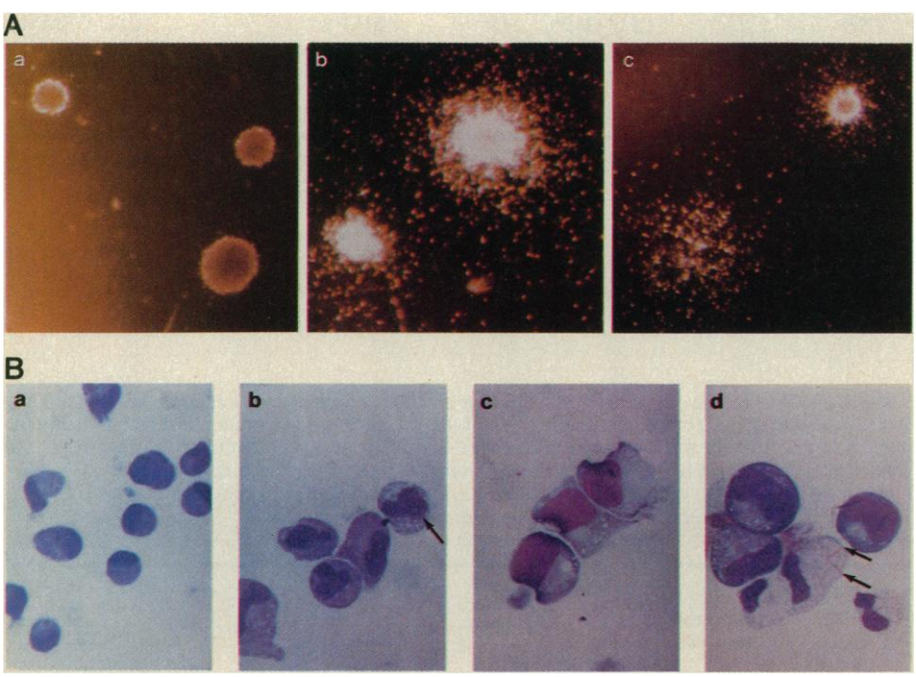

Fig. 4. (A) Induction of differentiation of a murine myelomonocytic leukemia cell line, WEHI-3B(D^+), by hG-CSF or rhG-CSF. Sixty WEHI-3B(D^+) murine myelomonocytic leukemic cells were plated in 0.2 ml of soft agar containing (a) medium, (b) 100 units of hG-CSF, or (c) 100 units of rhG-CSF as described (*35*). Leukemic cells form compact colonies of undifferentiated cells, but in the presence of hG-CSF the colonies are surrounded by dispersed migrating cells that have undergone differentiation to granulocytes and macrophages. (B) Induction of differentiation of human leukemic cells isolated from patients with promyelocytic leukemia (M3) and myeloblastic leukemia (M2) by hG-CSF or rhG-CSF. Light-density bone marrow cells, containing greater than 70 percent blasts or promyelocytes, were obtained from two patients with acute nonlymphocytic leukemia, FAB (French-American-British) classifications M3 (a and b) and M2 (c and d), respectively. Initial bone marrow cells from these patients were subjected to a density reduction with Ficoll-Hypaque (density, 1.077 g/cm^3; Pharmacia), and the low-density cells were resuspended in Iscove's modified Dulbecco's medium, at 1×10^6 cell/ml containing 10% fetal calf serum and 1% L-glutamine. Cells were cultured in (a and c) medium alone or (b and d) in the presence of rhG-CSF (1×10^5 unit/ml) and assayed for evidence of morphological differentiation on day 4 of culture. Arrows point to auer rods (light microscopy, ×1250).

proliferation of a number of different hematopoietic progenitors (*15, 16, 20*) and to activate neutrophils to kill targets through the mechanism of antibody dependent cell-mediated cytotoxicity (ADCC) (*38*) may prove valuable in treating immune compromised patients, bone marrow transplant patients, and various types of anemia. The ability of some myeloid leukemias to proliferate has been observed to coincide with the constitutive capacity to synthesize CSF (*39, 40*). In addition, human pluripoietin α (a GM-like CSF) is a potent stimulator of leukemic cell proliferation (*41*). The data on binding of hG-CSF to new clinical isolates of human leukemic cells and the murine myelomonocytic cell line presented here, and presented elsewhere for CSF-β (*19*), suggest that hG-CSF may have the potential to inhibit myeloid leukemia cell growth by induction of terminal differentiation. The factor with analogous differentiation activity in the murine system, G-CSF, profoundly inhibits the leukemogenicity of treated murine myeloid leukemic cells (*42, 43*). When attempts are made to differentiate leukemic cells in vivo it will be important to maintain the normal hematopoiesis and activate effector functions of mature cells. Thus, further studies will be necessary of cells from a large number of patients with newly diagnosed leukemias to determine which classes of myeloid leukemias respond to hG-CSF by differentiation and not by proliferation.

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21. Supernatant fluids were prepared from 5637 (1A6) cells grown in roller bottles (Corning 900 cm^2) containing glass microcarriers. Briefly, roller bottles were conditioned for 24 hours with spent medium (Iscoves containing 10 percent fetal calf serum (FCS), 2 m*M* glutamine, 100 unit/ml of penicillin, and 100 μg/ml of streptomycin) from flasks of 1A6 cells, then seeded at 10 to 20 percent confluency. The next day, after the cells had spread out, 0.8 to

1.0 g of sterilized glass microcarriers were added and the cells were allowed to attach for 4 hours; additional 1A6 cells were then added. The cells were grown to 40 to 50 percent confluency, then placed on a low serum containing medium (0.2 percent FCS) for 48 hours. The medium was harvested and the cells placed back in standard serum for 48 hours, after which there was one more cycle of harvest. The hG-CSF was then purified as described (*15*) except for the modification of the final step mentioned in the text.
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27. Bacterial cultures were grown with shaking in 2-liter flasks containing 500 ml of 2× TYE (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) at 30°C until the absorbance was 0.5 at 600 nm. The temperature was rapidly shifted to 42°C and the cultures were incubated an additional 3 to 3.5 hours. The cells were harvested by centrifugation at 5000*g* for 20 minutes in a Beckman JA10 rotor. Four grams of cells were resuspended in 25 ml of water and lysed by multiple passages through a French Press (10,000 pounds per square inch). The broken cell suspension was centrifuged at 10,000*g* for 10 minutes in a Beckman JA20 rotor and the pellet resuspended at approximately 5 to 6 mg/ml in water. The material was then solubilized in 1 percent sodium laurate, 50 m*M* tris, *p*H 8.5, and 5 percent ethanol (approximately 1 mg/ml). The solubilized pellet was centrifuged at 15,000, for 5 minutes and the supernatant loaded on a C_4 HPLC column. The C_4 column was washed in buffer A (100 m*M* ammonium acetate, *p*H 6.0 to 7.0) and a 2-propanol gradient (0 to 80 percent in buffer A) was run at a flow rate of 1 ml per minute collecting 1-ml fractions. The rhG-CSF eluted at fractions 71 to 73.
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Electrophoretic Separations of Large DNA Molecules by Periodic Inversion of the Electric Field

GEORGES F. CARLE, MARK FRANK, MAYNARD V. OLSON

In gel electrophoresis, nucleic acids and protein-detergent complexes larger than a threshold size all migrate at the same rate. For DNA molecules, this effect can be overcome by the simple procedure of periodically inverting the electric field. Tuning the frequency of the field inversions from 10 to 0.01 hertz, makes it possible to resolve selectively DNA's in the size range 15 to >700 kilobase pairs.

GEL ELECTROPHORESIS, IN WHICH ions are separated by size as they migrate through a gel medium in response to an applied electric field, is widely used to separate complex mixtures of macromolecules (*1*). Although it has been successfully adapted to an enormous range of biochemical applications, electrophoresis remains subject to a number of significant practical limitations. For example, in conventional electrophoretic separations of native DNA molecules on agarose gels, the largest molecules that can be separated readily are approximately 15 to 20 kb (~10^7 daltons) (*2*); larger molecules exhibit nearly size-independent mobilities, a phenomenon that is thought to be associated with an end-on, reptile-like mode of migration known as "reptation" (*3*). In 1983, Schwartz *et al.* addressed this problem by employing a complex electrode geometry that allowed alternate application of two transverse electric fields that were spatially inhomogeneous (*4*). The idea behind this method was to force large molecules to turn corners periodically in the gel matrix, thereby introducing a source of size-dependence that is absent during simple migration in one dimension. Various implementations of this technique have been used to study many previously uncharacterized DNA molecules, including the intact chromosomal DNA molecules of yeast and several protozoans (*5–8*).

In attempting to make further improvements in large-DNA electrophoresis, we have experimented with simpler electrode geometries than those employed either by Schwartz *et al.* (*4, 6*) or in our own implementation of "orthogonal-field-alternation gel electrophoresis" (OFAGE) (*5*). These experiments have led to the unexpected discovery that the electrophoresis of large DNA molecules can be made strongly size-dependent simply by periodically inverting a uniform electric field in one dimension. Typical results for molecules in the size range 2 to 400 kb are shown in Fig. 1. In these experiments, for which we used 1 percent agarose gels at a voltage gradient of 10.5 V/cm, net migration in the "forward" direction was achieved when a longer portion of each switching cycle was set for "forward" than for "reverse" migration.

In a control experiment in which only forward electrophoresis was carried out, we obtained good fractionation of molecules up to about 15 kb, slight fractionation in the 15- to 100-kb range, and size-independent mobilities for molecules >100 kb (Fig. 1A). When the switching cycle involved a forward interval of 0.5 second followed by a reverse interval of 0.25 second (Fig. 1B), we obtained a "window" of good fractionation between 15 to 30 kb, while the largest molecules still comigrated. The window of enhanced resolution can be shifted to the 50- to 125-kb region by lengthening the switching cycle (Fig. 1C). Similar results were obtained with a constant switching interval in combination with different forward and reverse voltages (*9*).

The experiment in Fig. 1C also illustrates a common feature of field-inversion gel electrophoresis (FIGE): the mobility goes through a minimum as a function of size; in this case, the minimum mobility lies in the neighborhood of 200 kb. The existence of a minimum mobility is demonstrated by the slower mobility of T4 DNA compared both to T5 DNA and to the mixture of yeast chromosomal DNA's; furthermore, in the yeast sample, the faint bands that trail behind the main front arise from the smaller yeast chromosomes, whose sizes are in the range of 260 to 290 kb (*5*).

In larger size ranges, the existence of a domain in which mobility increases with

Department of Genetics, Washington University School of Medicine, St. Louis, MO 63110.