SYNTHESIS OF GRANULOCYTE COLONY-STIMULATING FACTOR AND ITS REQUIREMENT FOR TERMINAL DIVISIONS IN CHRONIC MYELOGENOUS LEUKEMIA

By HELGA KLEIN,* REINHARD BECHER,‡ MICHAEL LÜBBERT,* WOLFGANG OSTER,§ ENGELHARD SCHLEIERMACHER,‖ MARION A. BRACH,* LAWRENCE SOUZA,¶ ALBRECHT LINDEMANN,* ROLAND H. MERTELSMANN,* AND FRIEDHELM HERRMANN*

*From the *University of Freiburg, Department of Hematology and Oncology, D-7800 Freiburg; ‡University of Essen, Department of Internal Medicine, Tumor Research, D-4300 Essen; §Behringwerke AG, D-3550 Marburg; ‖University of Mainz, Institute for Anthropology, D-6500 Mainz, Federal Republic of Germany; and ¶Amgen Incorporated, Thousand Oaks, California 93120*

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Chronic myelogenous leukemia (CML) is a clonal disorder believed to originate in a primitive hematopoietic stem cell. During the initial course, leukemic cells in CML retain the capacity to differentiate and to mature. This period, referred to as stable phase (SP), antedates an acute blastic phase (blast crisis; BC), which is associated with loss of ability of leukemic cells to undergo differentiation and maturation. The primary defect that results in an increased mass of myeloid cells is not completely understood. It has been suggested, however, that not an unregulated proliferation but rather a discordant maturational process may contribute to the abundant production of mostly mature myeloid elements in SP CML (1). Since the proliferative potential of myeloid progenitor cells (MPC) from SP CML patients has shown to be impaired compared with the proliferative capacity of MPC derived from normal human bone marrow (2), it has been suggested that in CML the majority of the malignant cells could have been expanded in later maturational compartments (i.e., promyelocytes/myelocytes) (1). Recently, it has been shown that production of growth factors occurs in some malignant populations of both myeloid (3, 4) and lymphoid (5) neoplasms, leading to autonomous growth. This study was therefore designed to investigate whether similar mechanisms might also contribute to the expansion of the myeloid compartment in CML.

Materials and Methods

RNA and DNA Analysis. Total cytoplasmic RNA from freshly isolated low density bone marrow-derived leukemic cells of 54 patients with CML was hybridized to a ^{32}P-labeled granulocyte CSF (G-CSF) cDNA probe (6). RNA extraction was performed according to a previ-

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Address correspondence to Dr. F. Herrmann, Department of Hematology and Oncology, University of Freiburg, Hugstetter Str. 55, D-7800 Freiburg i.B., FRG.

ously described protocol (7). In addition to probing for G-CSF, in selected cases RNA was also probed with cDNA specific for IL-3 and granulocyte/macrophage CSF (GM-CSF) (provided by D. Krumwieh, Behringwerke, Marburg, FRG), M-CSF (provided by P. Ralph, Cetus Corp., Emeryville, CA), and IL-6 (provided by T. Hirano, Osaka University, Osaka, Japan). In addition, RNA obtained from subpopulations of SP CML blood cells was analyzed. In all cases RNA was also hybridized to an α-actin cDNA (kindly provided by R. J. Schwartz, Houston, TX) to control for RNA loading. DNA blotting was performed after restriction endonuclease cutting of DNA derived from bone marrow samples of all 54 patients with the enzymes Hind III, Bgl II, Bam HI, Eco RI as described (8). As germline control DNA derived from normal bone marrow–derived low density cells was used. All blots were rehybridized to a 2-kb Bgl II/Hind III 5′ *bcr* probe (kindly provided by C. Bartram, Ulm, FRG) to exclude incomplete digestion or overdigestion and showed rearranged *bcr* sequences.

Purification of Subsets of Cells from CML Blood. The method of purification of various subpopulations of cells from CML blood has been previously reported (9) involving the use of a panel of mAbs (10) and an immune rosette technique to positively or negatively select for CML blood cell subsets. mAbs used are directed against the following antigens: CD2, CD3, (T cells), CD10, CD19, CD20 (B cells), CD16 (NK cells and mature neutrophils), CD11b (pan myeloid), CD14 (monocytes), CD33 (early myeloid), CD34 (hematopoietic progenitor cells), HLA-DR, glycophorin A (nucleated red cells). To prepare blast cell-enriched fractions (Be-F), PMNC were depleted from CD2, 3, 10, 11b, 16, 19, 29, glycophorin A-positive cells. Promyelocyte/myelocyte-enriched fractions (P/M-eF) were obtained following depletion of HLA-DR, CD2, 3, 10, 16, 19, 20, 34, glycophorin A, CD16-positive cells and subsequent selection of $CD33^+$ positive cells. To prepare granulocyte-enriched fractions (GRAN) dextran-sedimented PBC were positively selected for $CD16^+$ cells.

Detection of Biologically Active G-CSF in Culture Supernatants of Subsets of Cells from CML Blood. Secretion of the G-CSF protein by PBC-derived leukemic cell subpopulations was assayed in a standard two-layer agar (bacto-agar, Difco Laboratories, Detroit, MI) normal bone marrow day 7 CFU-G assay (10) in the presence or absence of 750 neutralizing U/ml of a mAb to G-CSF (neutralizing concentrations of all anticytokine mAbs used in this study were defined by the manufacturers). To this end, individual cell fractions of three stable phase CML patients were cultured at 10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS. After 48 h of culture, cell-free culture supernatants were harvested and incorporated (15% vol/vol) into a CFU-G assay (9, 10).

Results and Discussion

Leukemic cells were obtained before chemotherapy in 54 patients with CML, 27 diagnosed as having stable phase disease and 27 with myeloid blastic phase. In leukemic cells of all patients the Philadelphia chromosome (Ph^1) abnormality (11) and rearranged c-*abl/bcr* sequences (12) were detected (not shown). In five of nine patients with acute blastic phase, an additional chromosome abnormality, an isochromosome 17q [i(17q)] disclosed clonal evolution. RNA was extracted from leukemic populations of bone marrow specimens of all 54 patients and hybridized to G-CSF–specific cDNA (Fig. 1). Accumulation of G-CSF transcripts was detected in 10 of 27 SP samples (Fig. 1 *A*, *bottom panel*) and in 4 of 27 acute blastic phase samples, two of whom had i(17q). To define the cell population responsible for the G-CSF synthesis by SP CML cells, a first set of experiments was performed analyzing RNA from peripheral blood cells for G-CSF transcripts in comparing with transcripts of G-CSF previously obtained from bone marrow samples. In these experiments (not shown), G-CSF mRNA was also detectable in five peripheral blood–derived leukemic samples of five different samples investigated, thus excluding the possibility of G-CSF synthesis by mesenchymal bone marrow cells. In additional experiments (Fig. 2), density separation

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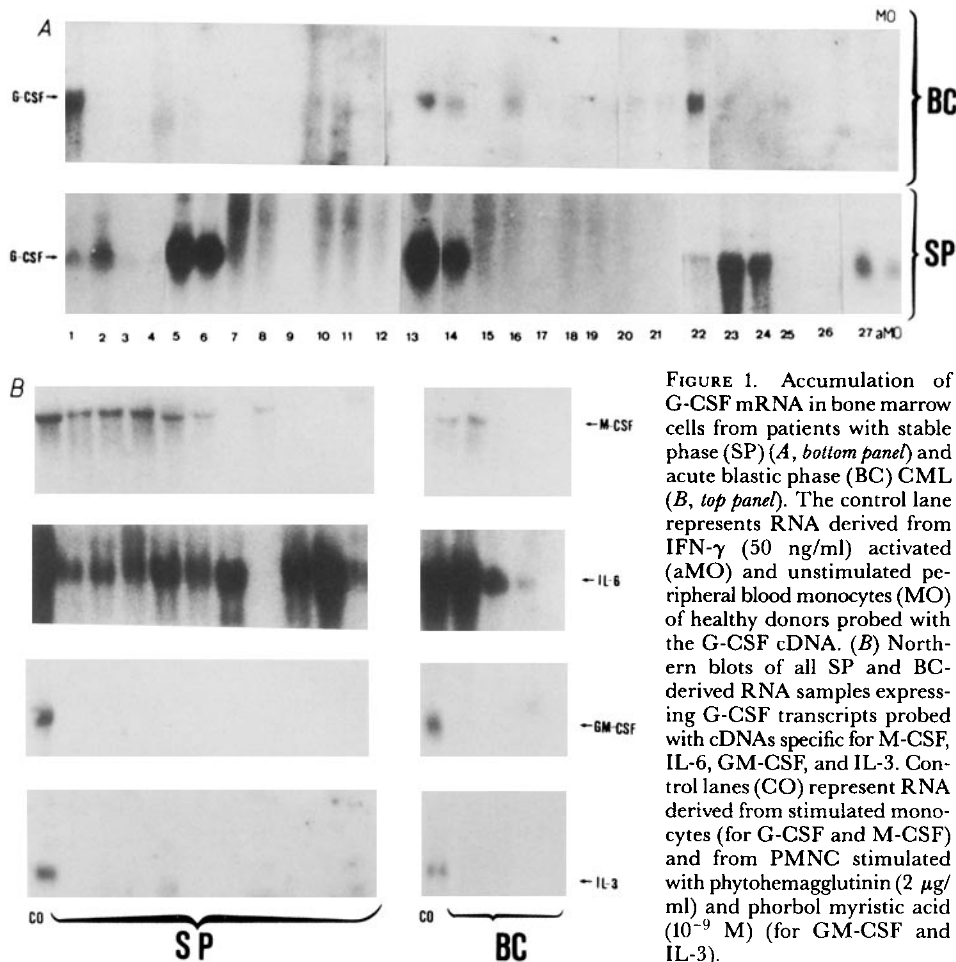

FIGURE 1. Accumulation of G-CSF mRNA in bone marrow cells from patients with stable phase (SP) (*A*, *bottom panel*) and acute blastic phase (BC) CML (*B*, *top panel*). The control lane represents RNA derived from IFN-γ (50 ng/ml) activated (aMO) and unstimulated peripheral blood monocytes (MO) of healthy donors probed with the G-CSF cDNA. (*B*) Northern blots of all SP and BC-derived RNA samples expressing G-CSF transcripts probed with cDNAs specific for M-CSF, IL-6, GM-CSF, and IL-3. Control lanes (CO) represent RNA derived from stimulated monocytes (for G-CSF and M-CSF) and from PMNC stimulated with phytohemagglutinin (2 μg/ml) and phorbol myristic acid (10^{-9} M) (for GM-CSF and IL-3).

and a panel of mAbs followed by immune rosetting were used to obtain cell subpopulations that were enriched for promyelocytes/myelocytes (≥97% pure), for mature granulocytes (≥99% pure), and for blast cells (≥94% pure) by positive and negative selection procedures. G-CSF cDNA that was hybridized to RNA derived from enriched populations failed to detectably hybridize with blast cell–derived RNA. Specific signals, however, appeared when RNA from P/M-eF (giving the strongest signal) and GRAN (giving the weakest signal) were investigated (Fig. 2 *B*). Similarly, supernatants of cultures of blood mononuclear cells and their promyelocyte/myelocyte and granulocyte subpopulations, but not blast cell fractions, displayed biologic activity for G-CSF when tested on purified normal bone marrow–derived granulocyte colony-forming (CFU-G) cells (Fig. 2 *A*). To investigate whether maturing leukemic cells producing G-CSF also responded to this factor by stimulating their own growth in an autocrine fashion, SP CML cells enriched for promyelocytes/myelocytes were cultured in semisolid agar in the presence or absence of neutralizing

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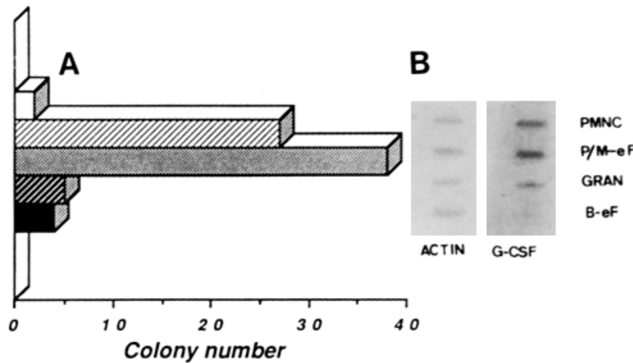

FIGURE 2. Expression of the G-CSF mRNA in subsets of PBC-derived leukemic cells of a patient (patient 14, see Fig. 1 *A*, *bottom panel*) with SP CML. The open bar represents a control in which CFU-G growth was stimulated with medium only. The other bars correspond to the slots shown in *B*. α-Actin cDNA controls for RNA loading. Identical experiments were performed with subsets of PBC-derived leukemic cells of two other patients (patients 5 and 6, see Fig. 1 *A*, *bottom panel*) and showed comparable results.

and nonneutralizing mAbs to recombinant human (rh) G-CSF and were investigated for their ability to generate clones. Fig. 3 shows that P/M-eF of CML populations generated up to 60 clones/10^3 cells over a period of 7 d, which could be inhibited by treating the cultures with neutralizing mAb to G-CSF, thus demonstrating that CML-derived promyelocytes/myelocytes use autocrinously secreted G-CSF to spur their own terminal divisions. It should be noted that anti-G-CSF antibody had no inhibitory effect on clonal growth of CML cultures established with cells not ex-

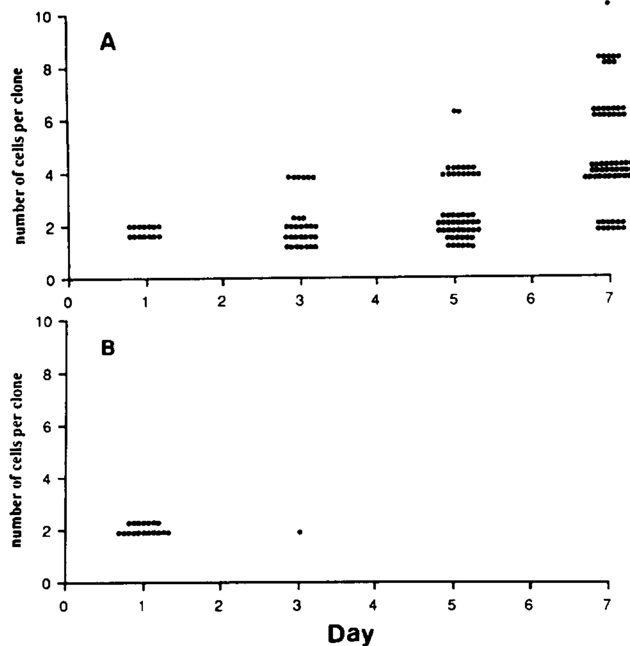

FIGURE 3. In vitro generation of clones by promyelocyte/myelocyte enriched fractions of PMNC (SP CML patients) in semisolid agar (patient 14, Fig. 1 *A*, *bottom panel*). The figure shows size distribution of clones generated over a culture period of 7 d. In *A* cells were cultured in medium only. In *B* the cultures were treated with a neutralizing mAb to rhG-CSF. Treatment of cultures with nonneutralizing mAbs to rhG-CSF, or neutralizing mAbs to M-CSF and IL-6 did not abrogate the generation of clones (not shown). In all experiments cells were cultured at 1,000 cells/ml in a mixture of 1 vol of double-strength IMDM, 1 vol of FCS, and 2 vol of 0.6% bacto-agar in 24-well plates (Linbro). The cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. Replicate cultures were scored at intervals between days 1 and 7 of incubation using a dissection microscope as described (13).

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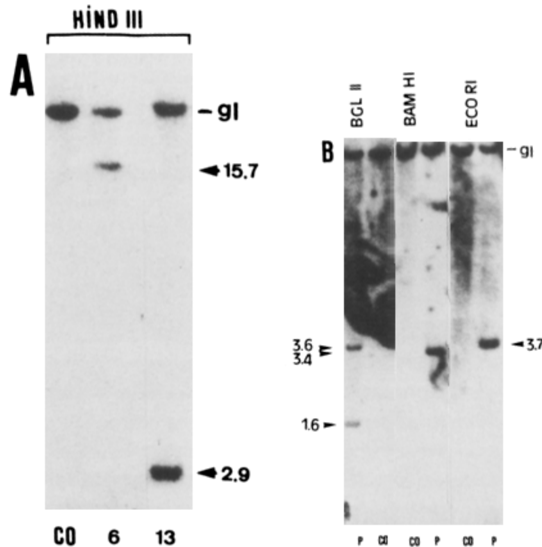

FIGURE 4. Southern blot analysis of DNA digested with Hind III (*A*) derived from low density bone marrow cells of two patients (patients 6 and 13) with blastic phase CML probing with a G-CSF cDNA. (*B*) DNA blots of DNA from low density bone marrow cells of patient 13 digested with Bgl II, Bam HI, and Eco RI, probed with a G-CSF cDNA (P). As germline control (CO) DNA derived from normal bone marrow low density cells was used. Incomplete digestion or overdigestion was excluded by demonstrating rearranged *bcr* sequences following rehybridization with a specific probe (not shown).

pressing the G-CSF gene. Since both SP and BC CML that expressed G-CSF also accumulated transcripts of M-CSF (9 of 14), IL-6 (12 of 14), but not of GM-CSF and IL-3 (Fig. 1 *B*), identical experiments were performed using neutralizing concentrations of mAbs to rh M-CSF (kindly provided by P. Ralph) and to rhIL-6 (kindly provided by T. Hirano). Anti-M-CSF and anti IL-6 mAbs were, however, unable to prevent generation of clones by P/M-eF of SP CML cells. Of the four G-CSF mRNA expressing BC CML samples investigated, only one grew autonomously in agar (patient 13, see Figs. 1 *A*, *top panel*; and 4, *A*, *B*). However, none of the anticytokine mAbs used (anti-G-CSF, -M-CSF, -IL-6) were capable of inhibiting autonomous blast growth, even when used in high concentrations (2,000 neutralizing U/ml) and in combinations (data not shown).

Normal blood or nonmesenchymal bone marrow cells do not secrete hematopoietic growth factors unless activated (3, 4); thus, constitutive expression of G-CSF by CML cells must be ranked as highly abnormal. Recently, rearrangement of the gene for GM-CSF has been shown to result in expression of the GM-CSF gene in some cases of acute myelogenous leukemia (14). Therefore, we searched for genomic alterations of the G-CSF, M-CSF, and IL-6 gene in all our patients. Restriction endonuclease analysis and Southern blotting revealed germline configurations of the G-CSF gene for DNA samples of 52 patients and of the M-CSF and IL-6 gene in all 54 patients (data not shown). Nongermline G-CSF gene-related fragments of variable sizes were, however, detected in two of nine cases of BC CML with i(17q), the chromosome to which the G-CSF gene had been assigned (15), thus suggesting that the i(17q) may occasionally bear a breakpoint within the G-CSF gene. Further work to analyze this chromosome region is being actively pursued in our laboratory.

Summary

In this paper we demonstrate that maturing neoplastic cells from patients with chronic myelogenous leukemia (CML) constitutively produce G-CSF and are also

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receptive for this molecule. G-CSF functions as an autocrine growth factor in stable phase CML, and thus is responsible for divisions of maturing leukemic cells leading to an expansion of the compartment of mature cells. This observation is well in line with in vivo features of CML in stable phase, i.e., the hyperplasia of the mature granulocyte compartment. In acute blastic phase of CML expression of the G-CSF gene seems to be less common and not related to autonomous blast growth.

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References

1. Strife, A., and B. Clarkson. 1988. Biology of chronic myelogenous leukemia: is discordant maturation the primary defect? *Semin. Hematol.* 25:1.
2. Goto, T., M. Nishikori, and Z. Arlin. 1982. Growth characteristics of leukemic and normal hematopoietic cells in Ph+ chronic myelogenous leukemia. *Blood.* 59:793.
3. Young, D. C., K. Wagner, and J. D. Griffin. 1987. Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. *J. Clin. Invest.* 79:100.
4. Oster, W., N. A. Cicco, H. Klein, T. Hirano, T. Kishimoto, R. Mertelsmann, and F. Herrmann. 1989. Participation of the monokines IL-6, TNF-alpha, and IL-1 beta secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. *J. Clin. Invest.* 84:451.
5. Kawano, M., T. Hirano, T. Matsuda, T. Taga, Y. Horii, K. Iwato, H. Asaoku, B. Tang, O. Tanabe, H. Tanaka, A. Kuramoto, and T. Kishimoto. 1988. Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. *Nature (Lond.).* 332:83.
6. Souza, L., T. C. Boone, J. Gabrilove, P. H. Lai, K. M. Zsebo, D. C. Murdock, V. R. Chazin, J. Bruszewski, L. Lu, K. K. Chen, J. Barendt, E. Platzer, M. A. S. Moore, R. Mertelsmann, and K. Welte. 1986. Recombinant human granulocyte colony-stimulating factor: Effects on normal and leukemic myeloid cells. *Science (Wash. DC).* 232:62.
7. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate phenol-chloroform extraction. *Anal. Biochem.* 162:156.
8. Southern, E. 1979. Gel electrophoresis of restriction fragments. *Methods Enzymol.* 68:156.
9. Cannistra, S. A., F. Herrmann, R. Davis, K. Nichols, and J. D. Griffin. 1986. Relationship between HLA-DR expression by normal myeloid progenitor cells and inhibition of colony growth by prostaglandin E. *J. Clin. Invest.* 77:13.
10. Herrmann, F., R. E. Schmidt, J. Ritz, and J. D. Griffin. 1987. In vitro regulation of human hematopoiesis by natural killer cells: analysis at the clonal level. *Blood.* 69:246.
11. Nowell, P. C., and D. A. Hungerford. 1960. Chromosome studies on normal and leukemic human leukocytes. *J. Natl. Cancer Inst.* 25:85.
12. Shtivelman, F., B. Lipschitz, R. P. Gale, and E. Canaani. 1985. Fused transcripts of abl and bcr genes in chronic myelogenous leukemia. *Nature (Lond.).* 315:550.
13. Begley, C. G., N. A. Nicola, and D. Metcalf. 1988. Proliferation of normal human promyelocytes and myelocytes after a single pulse stimulation by purified GM-CSF and G-CSF. *Blood.* 71:604.
14. Cheng, G. Y. M., C. A. Kelleher, J. Miyauchi, C. Wang, G. Wong, S. C. Clark, E. A. McCulloch, and M. D. Minden. 1988. Structure and expression of genes of GM-CSF and G-CSF in blast cells from patients with acute myeloblastic leukemia. *Blood.* 71:204.
15. Simmers, R. N., F. Smith, M. F. Shannon, O. M. Garson, G. Wong, M. A. Vadas, and G. R. Sutherland. 1987. Localization of the human G-CSF gene on chromosome 17 proximal to the breakpoint in the t(15;17) in acute promyelocytic leukemia. *Blood.* 70:330.

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