Stimulation of Nonclonal Hematopoiesis and Suppression of the Neoplastic Clone After Treatment With Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor in a Patient With Therapy-Related Myelodysplastic Syndrome

By Saroj Vadhan-Raj, Hal E. Broxmeyer, Gary Spitzer, Anne LeMaistre, Susan Hultman, Gerard Ventura, Jean-Dominique Tigaud, Marjorie A. Cork, Jose M. Trujillo, Jordan U. Guterman, and Walter N. Hittelman

A complete hematologic remission was achieved in a patient with therapy-related preleukemia and transfusion-dependent pancytopenia after treatment with recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF). The patient remained in remission for nearly 1 year despite the discontinuation of GM-CSF treatment. Several lines of evidence suggest that normal hematopoiesis was restored after GM-CSF treatment. First, the cytogenetic anomaly, which was present before GM-CSF, completely disappeared after three cycles of treatment. Cytogenetic conversion was documented by conventional karyotypic evaluation of mitotic bone marrow cell preparations as well as by premature chromosome condensation analysis of the nonmitotic cells of bone marrow and peripheral blood. Second, the growth pattern and cycle status of bone marrow granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) progenitor cells were found to be normal during remission. Third, X chromosome-linked restriction fragment length polymorphism-methylation analysis of DNA from mononuclear cells (>80% lymphocytes) and mature myeloid elements showed a polyclonal pattern. These findings suggest that restoration of hematopoiesis in this patient after GM-CSF treatment may have resulted from suppression of the abnormal clone and a selective growth advantage of normal elements.

THERAPY-RELATED myelodysplastic syndrome and acute nonlymphocytic leukemia (ANLL) are serious hematopoietic disorders that may occur as a late complication of treatment with cytotoxic agents for neoplastic and nonneoplastic conditions. Compared with primary myelodysplastic syndromes and de novo ANLL, therapy-related myelodysplastic syndromes are characterized by a higher frequency of profound bone marrow failure, trilineage dysplasia, and cytogenetic aneuploidies involving chromosomes 5 and/or 7.

Attempts to prevent leukemic transformation in the preleukemic phase have not proven successful. Further, the response of these patients to conventional antileukemic agents after progression to acute leukemia has been extremely poor due to a high frequency of deaths during the aplastic phase. Therefore, even if the leukemic clone is abolished by cytotoxic treatment, repopulation of the marrow with normal elements is infrequent, either due to the possibility that the normal elements have been replaced by clonal multipotent stem cells, or the likelihood that prior exposure to mutagens have compromised the capabilities of the normal residual elements to repopulate the marrow.

Most patients are elderly and thus not suitable candidates for human leukocyte antigen-matched bone marrow transplantation. Further, a substantial proportion of patients die from complications of cytopenia before the development of overt leukemia.

We and others have previously demonstrated the stimulatory effects of treatment with recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) on hematopoiesis in patients with myelodysplastic syndromes. However, despite the improvement in blood counts, the neoplastic clones persisted in the bone marrows. We have subsequently treated eight additional patients with myelodysplastic syndrome with GM-CSF and achieved a complete hematologic as well as cytogenetic remission in one patient with therapy-related preleukemia and pancytopenia. To elucidate the nature of this patient’s response, we studied the morphologic and cytogenetic characteristics of her mitotic and nondividing cells, the growth pattern and cycling rates of hematopoietic progenitor cells, and by DNA polymorphism-methylation analysis the clonal identity of peripheral blood and bone marrow cells. We now report that GM-CSF treatment was followed by suppression of the leukemic clone and stimulation of nonclonal hematopoiesis in this patient.

CASE REPORT

The patient, 62 years old, presented to the University of Texas M.D. Anderson Cancer Center in March 1977 at age 51 with stage III, grade III, poorly differentiated adenocarcinoma of the ovary. She underwent a total abdominal hysterectomy and bilateral salpingo-oophorectomy, followed by treatment with 10 courses of melphalan between April 1977 and June 1978. A second-look laparotomy in August 1978 revealed no gross disease, although microscopic washings were positive for residual ovarian carcinoma. The patient then continued treatment with 12 courses of melphalan from September 1978 to October 1979. She was periodically restaged clini-
cally and radiographically over the past 9 years and has remained free of disease.

In May 1987 the patient underwent a right hip joint replacement for severe debilitating rheumatoid arthritis; her blood counts were normal at that time. However, in August 1987 she began experiencing progressive fatigue and weakness. In September 1987 she had an extraction of an infected tooth. She sought medical attention subsequently because of persistent infection at the site of the tooth extraction and fatigue. At that time her blood workup revealed a hemoglobin level of 4.6 g/100 mL, WBC of 800/μL, absolute granulocyte count of 56/μL, and platelet count of 65,000/μL. A bone marrow aspirate showed 17.5% blasts and 4.5% promyelocytes. The marrow biopsy revealed 70% cellularity, with trilineage dysplasias consistent with the diagnosis of refractory anemia with excess blasts. Dental evaluation revealed advanced periodontal disease with severe bone loss, but she was considered a poor candidate for dental extraction because of severe pancytopenia. The patient received frequent blood transfusions and intravenous antibiotics for gram-negative sepsis in the ensuing weeks.

In an attempt to restore hematopoiesis, she was treated with recombinant human GM-CSF (specific activity, 5 × 10^7 units/mg of protein, Immunex Corporation, Seattle) in November 1987 as part of a phase I study of GM-CSF. She received 120 μg/m²/d by continuous intravenous infusion daily for 2 weeks, and this was repeated at about 2-week intervals. Treatment was associated in this patient with a multilineage hematopoietic response characterized by significant increases in WBC, granulocyte, platelet, and corrected reticulocyte counts and hemoglobin and hematocrit levels (Fig 1). After the third cycle, for which dose was reduced to 60 μg/m²/d, GM-CSF was discontinued. The patient underwent total dental extraction and alveoloplasty and became free of infections. Moreover, she remained in complete hematologic remission (ie, absolute granulocyte count >1,000/μL, hematocrit >30%, platelet count >100,000/μL, and bone marrow with blast count <5% and with normal morphology) for nearly 1 year (11.5 months) and did not require blood transfusions. Thirteen months after the initiation of GM-CSF treatment (10 months after the discontinuation of GM-CSF treatment) the patient showed signs of relapse with cytogenetic evidence of clonal evolution. Reinitiation of GM-CSF at this time did not reverse disease progression.

**MATERIALS AND METHODS**

Peripheral blood, bone marrow aspirates, and biopsy specimens were processed by conventional methods and evaluated by light and electron microscopy before and after each cycle of GM-CSF treatment. The percentage of blast cells in the bone marrow aspirates was determined by 500-cell differentials on Wright-Giemsa stained smears. Classification of myelodysplastic syndrome was made according to the French-American-British (FAB) criteria. For

---

Fig 1. Effects of recombinant human GM-CSF treatment on hematologic indexes in a patient with therapy-related preleukemia. (A) The effects on WBCs, granulocytes (AGC), and platelets. (B) The effects on hematocrit and corrected reticulocytes. Arrows denote transfusions of packed RBCs.
ultrastructural studies fresh peripheral blood cells and bone marrow aspirates were fixed in 2.5% glutaraldehyde, prepared by the standard techniques, and examined for morphology and presence of myeloperoxidase granules.

Assay for hematopoietic progenitor cells. The assays for granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) progenitor cells were performed as described previously. Briefly, bone marrow cells were separated into a low-density fraction using Ficoll-Hypaque and plated at 1 x 10⁶ cells/mL in agar culture medium with 10% (v/v) 5637 human cell-line conditioned medium (CM) or without exogenous growth factors. Colonies (>40 cells per aggregate) and clusters (3 to 40 cells per aggregate) derived from GM progenitor cells were scored after 7 and 14 days of incubation. The percentages of bone marrow CFU-GM and BFU-E in DNA synthesis (S-phase of cell cycle) were measured by the high-specific-activity tritiated-thymidine kill technique as previously described.

Cytogenetic studies. Chromosome studies were performed as previously described²⁻⁴ on bone marrow samples before treatment, after three treatment cycles, and at 3-month intervals thereafter. The karyotypes were reported according to the International System for Human Cytogenetic Nomenclature (ISCN 1985).

Premature chromosome condensation analysis. The karyotypes of both mitotic and nondividing bone marrow cells and postmitotic peripheral blood cells were determined using the technique of premature chromosome condensation, which allows visualization of interphase chromosomes. As described,²⁻⁴ bone marrow cells and peripheral blood cells were separated into a mononuclear cell fraction and polymorphonuclear cell fraction by two-step Ficoll-Hypaque gradient. Mitotic Chinese hamster ovary (CHO) cells were used to induce premature chromosome condensation in peripheral blood cells and bone marrow cells. The CHO cells were prelabeled with BrdU to distinguish their chromosomes from the prematurely condensed chromosomes (PCC) after differential staining of slides. The number of chromosomes in fused cells was determined, and the karyotype was determined using G-banding. The cell-cycle stage of the interphase cells at the time of fusion was determined by the morphology of the PCC as previously described.²⁻⁴ DNA studies for restriction fragment-length polymorphism-methylation analysis. Granulocytes and light-density mononuclear cells were isolated from peripheral blood and bone-marrow samples after centrifugation on Ficoll-Hypaque, and high molecular-weight DNA was obtained from both fractions and from the mature myeloid bone marrow fraction by sodium dodecyl sulfate-proteinase K digestion followed by phenol-chloroform extraction. Restriction-enzyme digestions were carried out as recommended by the suppliers of the enzymes (Promega and Boehringer Mannheim).

DNA hybridization studies. Ten micrograms of DNA of each cell fraction was digested with appropriate restriction enzymes overnight at 37°C as previously described.²⁻⁴ The digested DNA samples were electrophoresed through a 1.5% agarose gel, denatured, neutralized, and then transferred to nylon (Nytran) membrane filters. After baking in a vacuum oven for two hours at 80°C, the filters were prehybridized and then hybridized for 48 hours at 42°C in a solution containing 50% formamide in 5X SSC (0.75 mol/L NaCl, 0.075 mol/L sodium citrate, pH 7.4), 5X Denhardt's solution, 100 μg/mL of herring sperm DNA, and a phosphoglycerate kinase (PGK) probe labeled with 32P to a specific activity of 1 x 10⁶ CPM per μg of DNA. The PGK probe was a 800-base pair BamHI-EcoRI fragment derived from the 5' end of the PGK gene.²⁻⁴ Hybridized filters were washed at 60°C in 0.1X SSC and 0.1% SDS for 60 minutes. Filters were air-dried and autoradiographed at -70°C for 1 to 5 days.

RESULTS

Treatment of this patient with GM-CSF resulted in a multilineage response that was maintained for nearly 1 year despite a dose reduction in the third cycle (60 μg/m²/d) and discontinuation of GM-CSF treatment after three cycles (Fig 1). Furthermore, the leukocyte alkaline phosphatase activity (LAP score) was also found to be within normal limits during remission (Table I).

Bone marrow and peripheral blood morphology. Light and electron microscopic examination of the patient's bone marrow before GM-CSF treatment revealed dysplastic maturation in all cell lineages as described by FAB criteria.³ The granulocytes and megakaryocytes were decreased and no ringed sideroblasts were noted. The myelodysplastic syndrome was classified as refractory anemia with excess blasts (RAEB) due to the increased numbers (17.5%) of minimally differentiated myeloid blasts (FAB type I), some with nuclear blebs on ultrastructural studies, similar to those described in cases of acute undifferentiated leukemia.²⁻⁴ These blasts were minimally myeloperoxidase positive by light microscopy. The peripheral blood demonstrated pancytopenia, few circulating blasts, nucleated RBCs, and granulocytic hyposegmentation and hypogranularity. The rise in

<table>
<thead>
<tr>
<th>Time of Analysis (day)*</th>
<th>Baseline Pretreatment</th>
<th>Post Cycle 1 (18)</th>
<th>Pre Cycle 2 (46)</th>
<th>Post Cycle 2 (60)</th>
<th>Pre Cycle 3 (82)</th>
<th>Post Cycle 3 (97)</th>
<th>1 Month Post Study (126)</th>
<th>2 Months Post Study (166)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blasts (%)</td>
<td>17.50</td>
<td>6.30</td>
<td>1.00</td>
<td>0.40</td>
<td>1.30</td>
<td>0.50</td>
<td>0.80</td>
<td>1.60</td>
</tr>
<tr>
<td>Cellularity (%)</td>
<td>70</td>
<td>75</td>
<td>60</td>
<td>70</td>
<td>50</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Myeloid/erythroid cell ratio</td>
<td>0.80</td>
<td>0.46</td>
<td>0.43</td>
<td>0.65</td>
<td>1.19</td>
<td>1.17</td>
<td>1.48</td>
<td>1.24</td>
</tr>
<tr>
<td>Myeloid maturation index</td>
<td>1.27</td>
<td>4.47</td>
<td>9.91</td>
<td>26.00</td>
<td>15.14</td>
<td>24.11</td>
<td>26.89</td>
<td>15.20</td>
</tr>
<tr>
<td>LAP score‡</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>5</td>
<td>32</td>
<td>ND</td>
<td>57</td>
</tr>
<tr>
<td>GM-CSF dose (μg/m²/d)</td>
<td>120</td>
<td>120</td>
<td>60</td>
<td>32</td>
<td>ND</td>
<td>57</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>
peripheral blood counts after GM-CSF treatment was accompanied by a decrease in the percentage of bone marrow blasts, a decrease in dysplasia, and an increased proportion of mature myeloid elements, resulting in a marked increase in the bone marrow myeloid maturation index (Table 1). Interestingly, the overall cellularity decreased from 70% to 40%, but there was no evidence of selective suppression of nonmyeloid elements because their relative differential percentages remained constant. After three cycles of treatment, the patient's marrow morphology, cellularity (Table 1), and megakaryocyte number were within normal limits.

**Progenitor cell growth patterns.** In vitro culture of bone marrow cells during remission, after three treatment cycles, showed a normal growth pattern and cycle status of granulocyte-macrophage (with 10% (vol/vol) 5637 conditioned medium (CM), day 7 CFU-GM; 47 ± 1 colonies with 17% of cells in S-phase) and erythroid progenitor cells (with 10% (V/V) 5637 CM, day 14 BFU-E; 31 ± 3 colonies with 32% of cells in S-phase).

**Cytogenetic analysis.** Before GM-CSF treatment cytogenetic examination of the bone marrow revealed 15 metaphases, 11 of which showed a normal female karyotype (46,XX) and four a hyperdiploid clone [47,XX, +X, +2, dic(3q), −5,8q+,9p−, +11,12q−,14p+,17p+, −18] (Fig 2) consistent with the diagnosis of therapy-related preleukemia. After three cycles of treatment with GM-CSF, cytogenetic analysis of the bone marrow revealed a normal female karyotype in all 25 metaphases examined. Repeat examination 6 months later, two of 100 peripheral blood mononuclear cells examined showed 47 chromosomes. These findings suggested that the karyotypically abnormal clone was reduced to background levels after GM-CSF treatment.

**DNA polymorphism-methylation analysis.** The finding of conversion to diploidy suggested that the abnormal clone had been reduced to undetectable levels. It was also possible, however, that this patient had had two abnormal clones (one with diploidy and one with aneuploidy) before treatment, and that treatment had brought the patient back to a clonal hematologic remission. To distinguish between these two possibilities, we assessed cellular clonality during hematologic remission using X chromosome-linked restriction fragment length polymorphism-methylation analysis, a strategy developed by Vogelstein et al.22,23. Examination of premiotic (G2 phase) as well as nondividing cells (G1/G0 phase) in the bone marrow also revealed a diploid karyotype. In a repeat analysis 6 months later, two of 100 peripheral blood mononuclear cells examined showed 47 chromosomes. These findings suggested that the karyotypically abnormal clone was reduced to background levels after GM-CSF treatment.

**Premature chromosome-condensation analysis.** To determine the karyotype of the mature myeloid cells in peripheral blood (before and after treatment) and of the mitotic and nonmitotic cells from the bone marrow (after treatment), the premature chromosome condensation technique was used (Fig 3A). Before GM-CSF treatment, of 48 peripheral blood mononuclear cells examined, 15% exhibited a hyperdiploid clone with 47 chromosomes (Fig 3B), which by G-banding analysis, contained the same karyotypic changes found in the bone marrow preparations by conventional cytogenetic analysis. After three treatment cycles none of the 83 mononuclear cells examined exhibited 47 chromosomes (Fig 3B), whereas two of the 31 mature myeloid cells scored showed 47 chromosomes, which is within the background level for this technique.22,23 Examination of premiotic (G2 phase) as well as nondividing cells (G1/G0 phase) in the bone marrow also revealed a diploid karyotype. In a repeat analysis 6 months later, two of 100 peripheral blood mononuclear cells examined showed 47 chromosomes. These findings suggested that the karyotypically abnormal clone was reduced to background levels after GM-CSF treatment.

**Fig 2. Mitotic karyotype of patient's bone marrow cells before GM-CSF treatment.** Giemsa-banded chromosomes of bone marrow cells show a hyperdiploid clone [47,XX, +X, +2, dic(3q), −5,8q+,9p−, +11,12q−,14p+,17p+, −18].
with methylation analysis. Conversely, if DNA is derived from a polyclonal cell population, each of the two fragments will contain active and inactive X chromosomes, and methylation analysis will demonstrate the presence of both alleles.

Our patient was found to be heterozygous at the BstX1 site of the PGK gene (Fig 4, lanes 1, 3, and 5). Subsequent digestion with the methylation-sensitive enzyme HpaII showed both PGK fragments 1.05 and 0.90 kb (Fig 4, lanes 2, 4, and 6). The intensity of fragments was reduced by less than 30% to 70%, and the ratio of both fragments found in myeloid cells was similar to their ratio in mononuclear cells (>80% lymphocytes) used as normal controls (confirmed by densitometry). This finding was consistent with a polyclonal pattern as defined previously.25,26 A repeat examination 6 months later showed similar patterns. These results suggest that restoration of hematopoiesis after GM-CSF treatment resulted from stimulation of normal stem cells rather than clonal hemopathy.

**DISCUSSION**

The prior history of another malignancy with exposure to an alkylating agent, the presence of a hyperdiploid clone...
exhibiting a complex karyotype and an excess of blasts in the bone marrow, life-threatening cytopenia, and an episode of gram-negative sepsis portended a dismal prognosis for this patient. Treatment of this patient with GM-CSF was associated with a complete hematologic remission that was maintained even after discontinuation of this biologic agent. It was highly unlikely that this response represented a spontaneous remission unrelated to GM-CSF treatment considering that the patient had therapy-related preleukemia and that response was more pronounced with each repeated cycle. This response was of clinical benefit to this patient because she was then able to undergo dental surgery and was free of infections and blood transfusions for 1 year.

The pattern of response in this patient was in striking contrast to that observed in other patients with myelodysplasia who were treated with GM-CSF. In particular, her hematologic response was associated with normalization of the bone marrow morphology, megakaryocyte number, increase in mature granulocytic elements, a decrease in overall cellularity to normal, and normal growth patterns of progenitor cells. This result is distinct from the abnormal growth patterns observed in other myelodysplastic syndrome patients both before and after GM-CSF treatment. Moreover, the treatment was accompanied by a reversion from cytogenetic aneuploidy to diploidy. It is important to note that diploidy was observed in both the dividing cells as well as in the nondividing cells. This finding demonstrates that GM-CSF treatment induced a preferential stimulation of the normal elements such that the aneuploid clone was reduced to an undetectable level. Because a substantial proportion of patients with acute nonlymphocytic leukemia in complete hematologic and cytogenetic remission are known to exhibit clonal hematopoiesis, it was important to determine whether the diploid elements present in remission were derived from normal stem cells or were the result of a clonal hemopathy. Using X chromosome-linked DNA polymorphism-methylation analysis, we were able to demonstrate a polyclonal pattern in the DNA derived from mature myeloid cells as well as from her lymphoid elements, indicating that the hematopoiesis in remission was nonclonal. The degree of leukopenia in this patient unfortunately did not allow evaluation of clonality prior to GM-CSF treatment. However, based on our preliminary experience, polyclonality is rarely observed in other patients with myelodysplastic syndrome studied.

One intriguing possibility is that GM-CSF suppressed the abnormal clone in vivo by inducing terminal differentiation and thereby decreasing its potential for self-renewal. An important characteristic of preleukemia is impaired cellular maturation. The cellular maturation defect in this patient was evidenced by pancytopenia despite a hypercellular bone marrow and the absence of karyotypically abnormal cells in the mature granulocytic fraction before treatment. The above possibility is supported by in vitro findings in which induction of differentiation in HL-60 cells by GM-CSF and G-CSF was associated with reduced clonogenicity. Similarly, several recent studies in mice have demonstrated suppression of leukemogenic potential in some cases by inducing differentiation of specific clones of malignant cells with the in vivo use of GM-CSF or the differentiation-inducing protein MGI-2.

A second component of this patient’s response was stimulation of normal hematopoiesis. It has been previously shown that leukemic cells can overproduce such hematopoietic inhibitory substances as leukemia-associated inhibitory activity, identified as acidic isoferritin or leukemia-associated inhibitor (LAI), and that this overproduction can be suppressed by induction of differentiation. Thus, it is possible that GM-CSF-induced maturation of the abnormal clone in this patient allowed the previously inhibited normal elements to respond to this growth factor.

Although response of this patient was unusual compared with our previous experience, it was of interest because it demonstrated that GM-CSF has the potential of restoring hematopoiesis and suppressing leukemogenesis in occasional patients with preleukemic disorders. This observation, along with previous findings, suggests that GM-CSF elicits varying responses in different patients, including selective stimulation of normal stem cells with suppression of leukemic cells (as observed in this patient), stimulation of proliferation coupled with differentiation of both normal and abnormal elements (as observed in MDS patients with low leukemic infiltrate), or preferential proliferation of leukemic clones (as observed in a subset of patients with chronic myelogenous leukemia, RAEB in transformation, and acute myeloid leukemia). This variability in response probably reflects both the biologic characteristics of leukemic cells as well as leukemia-host interactions. It would therefore be of great importance if in vitro cellular (eg, the presence of growth-factor receptors), molecular (eg, degree of clonality), or functional (eg, responsiveness to CSF) assays could be devised, which could predict for the pattern of in vivo response in each patient. In some cases, therefore, GM-CSF might be used to restore hematopoiesis, and in others to recruit cells for subsequent cytotoxic treatment. In this way all different responses to GM-CSF might be exploited to possible therapeutic advantage.

ACKNOWLEDGMENT

The authors thank Mary Jo Kellagher, PA, for patient care, Garland Yee, Jeanne Rothberg, and Carol Hunter for technical assistance, and Maria Carballosa for preparing the manuscript.

REFERENCES

4. Pedersen-Bjergaard J, Osterlind K, Hansen M, Philip P,
40. Broxmeyer HE, Lu L, Bicknell DC, Williams DE, Cooper S, Levi S, Salfeld J, Arosio P: The influence of purified recombinant human heavy-subunit and light-subunit ferritins on colony forma-


Stimulation of nonclonal hematopoiesis and suppression of the neoplastic clone after treatment with recombinant human granulocyte-macrophage colony-stimulating factor in a patient with therapy-related myelodysplastic syndrome

S Vadhan-Raj, HE Broxmeyer, G Spitzer, A LeMaistre, S Hultman, G Ventura, JD Tigaud, MA Cork, JM Trujillo and JU Gutterman