Disappearance of Cytogenetic Abnormalities and Clinical Remission During Therapy With 13-Cis-Retinoic Acid in a Patient With Myelodysplastic Syndrome: Inhibition of Growth of the Patient's Malignant Monocytoid Clone

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Median survival is as little as 6 months for patients with refractory anemia with excess blasts who demonstrate an abnormal karyotype in the majority of marrow cells. We treated a patient who presented with 29% marrow blasts and 90% abnormal metaphases with 13-cis-retinoic acid. He achieved a complete clinical and cytogenetic remission during therapy. To determine the mechanism of the response, serial studies were done of the effects of 13-cis-retinoic acid and dexamethasone on in vitro growth of his marrow cells. During clinical remission, when the drug was not administered, marrow growth remained significantly depressed. During relapse, the remission growth pattern was replaced by overgrowth of the karyotypically abnormal monocytoid clone. Clonal growth occurred in cultures containing colony-stimulating activity or dexamethasone but was absent in cultures containing concentrations of 13-cis-retinoic acid achieved in vivo. After the drug was re instituted, a second clinical stabilization developed. Since 13-cis-retinoic acid inhibits normal monocyte colony growth, we postulate that the patient's unusual clinical responses to the drug were due to in vivo growth inhibition of the malignant monocytoid clone.

MYELODYSPLASTIC SYNDROME is characterized by abnormalities in cell proliferation leading to single or multiple cytopenias. It is a heterogenous group of diseases, encompassing patients with refractory anemia (RA), refractory anemia and excess blasts (RAEB), refractory anemia and excess blasts in transformation (RAEBT), acquired idiopathic sideroblastic anemia (AISA), and chronic myelomonocytic leukemia (CMML). It is thought to be due to clonal proliferation of an abnormal stem cell and is considered a preleukemic state because approximately 40% of patients progress into acute myelogenous leukemia. The median survival for a patient with excess marrow blasts or with a karyotype abnormality is 6 months.

13-cis-retinoic acid is a stereoisomer of retinoic acid, a normally occurring metabolite of vitamin A (retinol) that is clinically useful in patients with the myelodysplastic syndrome. It has been shown to affect normal and abnormal cell proliferation and differentiation in vitro and to prevent the development of radiation and carcinogen-induced tumors in vivo. Drug concentrations that are effective in vitro (10-7 to 5 x 10-9 mol/L) are achieved in vivo when the drug is administered orally at 3 mg/kg/d or 100 mg/m2/d (230 mg/d) doses commonly used in the clinical trials.

We report here the detailed studies of one individual who, 3 years ago, was found to have RAEB. An abnormal clone with a t(2:11) chromosome translocation was also identified at that time. He underwent a complete clinical and chromosomal remission during therapy with oral 13-cis-retinoic acid. Several months after administration of the drug was stopped, he relapsed. Administration of the drug was restarted, and he achieved a second clinical stabilization. His marrow growth patterns in vitro suggested that the clinical responses to oral 13-cis-retinoic acid and his prolonged survival were due to inhibition of proliferation of his malignant monocytoid clone.

CASE REPORT

A 61-year-old man with a 2-year history of an underlying arthritis and vasculitis (diagnosed by biopsy specimen of skin rash in 1980) was noted to have anemia, mild thrombocytopenia, and neutropenia with Pelger-Huët abnormality in the neutrophils (Fig 1). He had been treated with corticosteroids on several occasions with clinical improvement of his arthritis and skin rashes but not his hematologic problems. He also gave a history of exposure to various organic solvents and benzene in his work in tire manufacturing.

In Dec 1981, marrow studies revealed in 90% of the metaphases a t(2:11) (p21-22; q22-23) translocation. Therapy was initiated with 19-nor-testosterone decanoate, pyridoxine, and prednisone, but at month 6 because of worsening of his anemia, he was referred to the Medical College of Pennsylvania to enter the clinical trial of 13-cis-retinoic acid therapy. At the time of referral, he required two units of packed red blood cells monthly. His physical examination was unremarkable, and his blood counts after the transfusion revealed a hemoglobin level of 9 g/100 mL, hematocrit value of 25%, and WBC count of 5,200/μL with 69% bands, 28% lymphocytes, and 2% monocytes. His platelet count was 116,000/μL, and the reticulocyte count was 1.5%. His bone marrow was hypercellular (80%) with evidence of dysplasia in all cell lines. An increase in myeloblasts to 21% was observed. He was classified as having RAEB by the French-American-British recommendations. His cytogenetic studies again showed the translocation in 90% of the metaphases, with no other abnormality identified.

All medications except for maintenance doses of dexamethasone (3 mg/d) were discontinued 1 month before starting the clinical trial with 13-cis-retinoic acid. In month 7, 100 mg/m2/d (230 mg/d) 13-cis-retinoic acid therapy was initiated. At 10 months, the dexamethasone dose was reduced to 0.75 mg/d. Twelve months from his initial presentation (after 5 months of 13-cis-retinoic acid therapy), his blood counts had normalized, and he was transfusion independent. Except for an exacerbation of the rash and arthritis requiring a brief period of increased doses of dexamethasone he did well. By month 15, marrow blasts were decreased to 6%, but 60% of the

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Marrow metaphases still showed the abnormal clone. At that time the 13-cis-retinoic acid dose was halved because, in spite of changes in his diet, the patient's triglyceride levels had risen to more than 900 mg/dL and his cholesterol had risen above 400 mg/dL. In month 18 his bone marrow studies were normal. At that time, he did not demonstrate any abnormal metaphases, there were no excess marrow blasts, no Pelger-Huet–type cells were apparent, and his peripheral blood counts were normal. At month 22, because of continued clinical remission with fewer than 1% marrow blasts and concern over the long-term effects of the elevated lipids, the 13-cis-retinoic acid was discontinued after 16 months of 13-cis-retinoic acid therapy. One month later, marrow blasts remained <1%, but one of 18 metaphases examined was cytogenetically abnormal with the same t(2;11) translocation that was present at the time of diagnosis.

The patient remained in a clinical remission without receiving therapy until month 29 when the Pelger-Huet abnormality and dysplastic marrow changes returned: 40% of the marrow cells showed the abnormal karyotype, and marrow blasts had risen to 28%. At month 30, the WBC count was 2,400/µL with no normal granulocytes seen; the platelet count was 111,000/µL. 13-cis-retinoic acid therapy, 100 mg/m²/d (230 mg/d) was restarted, and the dexamethasone continued at 0.75 mg/d. He required six units of packed red blood cells in months 34 and 35 and two units in month 36. In months 34 and 36, 100% of the metaphases were abnormal and the marrow was hypercellular (88%) with marrow blasts of 12% and 29%, respectively. His WBC count had risen to 5,700/µL and platelet count to 133,000.

At month 40, 10 months after 13-cis-retinoic acid therapy had been restarted, however, his marrow cellularity had fallen to 45% with <20% blast forms seen. The t(2;11) translocation was still present in 100% of marrow metaphases. His WBC count was 6,800/µL with 45% bands and no pseudo–Pelger-Huet-type cells; the platelet count was 233,000/µL and the hematocrit value was depressed at 20. At month 42, the 13-cis-retinoic acid therapy was discontinued because abnormal liver function tests had developed.

MATERIALS AND METHODS

Marrow was obtained with informed consent from normal donors (8) and patient J.L. enrolled in the clinical trial of 13-cis-retinoic acid therapy. At each time point J.L. was studied, marrow from a normal donor was grown under the same culture conditions using the same stimulating materials as were used to study the patient.

Chemicals

Phytohemagglutinin (PHA HA-15, Burroughs-Wellcome, Greensville, NC) was reconstituted in distilled water and stored at −80 °C until used. 13-cis-retinoic acid (kindly provided by Hoffman-La Roche Co, Nutley, NJ) was stored in darkness at −20 °C and dissolved in ethanol for each experiment. Dexamethasone (a gift of Merck, Sharpe and Dohme, Inc, West Point, Pa) was dissolved in 75% ethanol. The final concentration of ethanol in the cultures did not exceed 0.1%.

Stimulating Materials

Media conditioned by normal human peripheral blood leukocytes exposed to 1% vol/vol PHA according to the method of Aye et al served as the source of granulocyte-monocyte colony-stimulating activity (CSA). The media were shown to be most active undiluted. 13-cis-retinoic acid (10⁻⁷ to 10⁻⁴ mol/L) and dexamethasone (10⁻⁶ to 10⁻⁵ mol/L) were included in the stimulating material alone and in combination in some dishes. These concentrations are those achievable by oral administration of the drugs.

Culture Techniques

Marrow was placed into semisolid agar cultures as previously described. Briefly, bone marrow aspirates from normal healthy donors or patients were drawn into syringes containing sterile preservative-free heparin, 250 µg/mL (GIBCO, Grand Island, NY). They were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), centrifuged at 1,000 g for 30 minutes, and the interface cells washed and suspended in RPMI 1640 medium supplemented with 15% fetal bovine serum, 20 mmol/L L-glutamine, 1% vitamins, and 1% penicillin/streptomycin (10,000 U/mL) (Flow Laboratories, Inc, McLean, Va). Marrow cells were thus washed free of plasma and drugs contained in the plasma.

Cells were added to a mixture of Iscove’s modified Dulbecco’s medium (IMDM) (GIBCO) or Dulbecco’s modification of Eagle’s medium (DMEM) (Flow) modified according to the method of Metcalf with 20% fetal calf serum (Hyclone Tissue Culture
Products, Logan, Utah) and 0.3% Bacto-Agar (Difco Laboratories, Detroit) to a final concentration of 100,000 cells/mL. Thereafter, 1 mL of the cell suspension was placed into 35-μL gridded Petri dishes (Lux Lab-Tek Division, Miles Laboratories, Naperville, III) to which 100 μL of stimulating material (see the preceding) had been added. Duplicate or triplicate cultures were done.

After gelling, the cultures were incubated for up to 14 days in either a 5% CO2 (for IMDM-containing cultures) or a 10% CO2 (for DMEM-containing cultures) fully humidified atmosphere at 37 °C. They were fixed with formal calcium, transferred to 3 x 2-in glass slides, dried under cellulose acetate filter papers (Schleicher and Shuell, Inc, Keene, NH), and then stained for chloroacetate and alpha naphthyl acetate esterases by the method of Yam et al as modified by Abrahm and Smiley.

Groups of greater than ten cells were considered clusters and groups of greater than 50 cells, colonies. Colonies were considered granulocytic if more than 90% of the cells in the colony contained chloroacetate esterase, monocytic if more than 90% of the colony cells contained alpha naphthyl acetate esterase, and mixed if neither enzyme predominated by 90%.

Methylcellulose stock (2.5%) for methylcellulose culture was prepared by the method of Worthen et al except that IMDM was used as the culture medium. Briefly, methylcellulose (Methocel Premium A4M, Dow Chemical Co, Midland, Mich) was dissolved in water at 90 to 100 °C and mixed upon cooling with an equal volume of IMDM prepared at 2x concentration. This mixture was stirred overnight at 4 °C. At the time of culture preparation, methylcellulose stock was mixed with the cell suspension and the appropriate stimulating material at room temperature to a final concentration of 1.2% methylcellulose and 100,000 cells/mL. One millilitre of the mixture was placed in each gridded Petri dish and incubated in a fully humidified 5% CO2 atmosphere for variable lengths of time. Quadruplicate cultures were performed for each culture condition.

Chromosome Studies

Marrow aspirates. Bone marrow specimens were processed on the day of aspiration and after 24 hours in culture and stained by the Trypsin-Giemsa–banding method as described. For each study, a total of 18 to 38 chromosome counts were done with at least 3 karyotype analyses. Periodically, studies were repeated to determine the effects of therapy.

In vitro culture. On day 8 or 9 of culture, individual plucked colonies or cells from the entire culture dish were washed free of methylcellulose in Hanks' balanced salt solution (GIBCO), placed into hypotonic solution, and further processed by standard methods for chromosome analysis.

Statistical Methods

Student's t test for paired samples was used.

Fig 2. Effect of 13-cis-retinoic acid (left panel) and dexamethasone (right panel) on the growth of GM-CFU from normal donors. Marrow cells from eight donors were cultured, fixed, and histochemically stained as described (Materials and Methods). Key: ●○●, granulocyte colonies + clusters/10⁵ cells plated; ○——○, monocyte colonies + clusters/10⁵ cells plated; II, SEM; *, P < .005; †, P < .025.

RESULTS

Response of Normal Marrow to Incubation With 13-Cis-retinoic Acid and Dexamethasone

Figure 2 illustrates the effect on normal myelopoiesis of 13-cis-retinoic acid and dexamethasone in the range of concentrations achieved by the patient during his therapy. The total number of granulocyte, monocyte, and mixed colonies is not significantly changed by either therapy or by a combination of the two drugs (data not shown).

However, as is shown, 13-cis-retinoic acid or dexamethasone at concentrations of 10⁻⁷ mol/L or greater significantly decreased the number of monocyte colonies (P < .005, except 10⁻⁶ mol/L dexamethasone, P < .025). Dexamethasone (10⁻⁷ and 10⁻⁵ mol/L) increased the number of granulocyte colonies (P < .025).

In Vitro Studies of Patient J.L.

Marrow from the patient was studied on seven occasions beginning after the completion of his first course of 13-cis-retinoic acid and continuing during the second period of 13-cis-retinoic acid administration (Fig 3). The number of granulocytic and mixed colonies and clusters is shown in the left panel, whereas that of monocytic is shown in the right. He was first studied in month 23 when 13-cis-retinoic acid therapy had been discontinued for 4 weeks. He was in a...
complete clinical remission with no abnormal metaphases found, normal marrow appearance, no Pelger-Huët-type cells seen on blood smear and normal peripheral blood counts. At that time, the only indication that he was not normal was his marrow growth pattern. The number of colonies grown by his marrow cells stimulated by CSA was only 10% of that of normal donor marrows. The proportion of granulocyte to monocyte colonies was similar to that seen in normals, and monocyte colonies were eliminated by $10^{-7}$ or $10^{-6}$ mol/L 13-cis-retinoic acid. He was still in clinical remission and not receiving therapy when he was next studied in month 26, but his marrow growth remained abnormal. He was now growing only one third of the number of colonies grown from normal donor marrow, and the proportion of monocyte to granulocyte colonies had become reversed from that seen in normal marrows. Inhibition of monocyte colony growth by dexamethasone was less than that seen in normal marrows, but 13-cis-retinoic acid again completely inhibited monocyte colony growth.

In month 29, after 13-cis-retinoic acid had been discontinued for 7 months, the patient was in relapse. Colony growth stimulated by CSA was similar to that seen in month 26, but at this time, monocyte colony growth was inhibited only by 13-cis-retinoic acid. In month 34, when 100% of the marrow metaphases showed the $(t;2;11)$ translocation, granulocyte colonies had disappeared whereas monocyte colony numbers had significantly increased. They were partially inhibited by dexamethasone, completely by 13-cis-retinoic acid. In month 36, dexamethasone no longer inhibited clonal growth, but it was completely inhibited by 13-cis-retinoic acid. Chromosome studies of cells from these monocytoid colonies demonstrated the $(t;2;11)$ translocation.

In month 37, no colonies grew under any culture conditions (data not shown). However, in month 40, when the peripheral blood smear had become more normal and the blast percentage and marrow cellularity had decreased, studies showed decreased numbers of monocyte colonies and the reappearance of granulocyte and mixed colonies and clusters.

**DISCUSSION**

As noted previously in studies by ourselves and others, 13-cis-retinoic acid inhibits monocyte colony growth in marrows from normal donors. In our previous studies we found that $5 \times 10^{-6}$ or $10^{-5}$ mol/L 13-cis-retinoic acid increased granulocyte colony numbers to greater than those seen in control cultures. However, in those studies and the current ones, we saw no increase when using less than or equal to $10^{-6}$ mol/L 13-cis-retinoic acid when cultures were examined at ten or 14 days of incubation. We cannot explain why our studies differ from those that found that $3 \times 10^{-7}$ or $10^{-6}$ mol/L 13-cis-retinoic acid increased GM-CFU 100% or 50%, respectively. Our findings do agree with those of several other investigators. Differences in CSA source (Mo cell– or giant cell tumor (GCT)–conditioned medium), in culture techniques, in methods of counting colonies, or in the day of incubation at the time of count may account for the differences observed.

Abnormal growth in vitro, as was demonstrated in the marrow from this patient, has been reported in up to half the patients suffering from myelodysplastic syndrome, though one study reported equivalent growth. The present report is the first study to show that even when a patient was apparently normal and in a complete clinical and chromosomal remission, the marrow growth pattern remained abnormal.

In addition, this is the first reported RAEB patient with a karyotype abnormality in whom oral administration of 13-cis-retinoic acid was associated with a complete cytogenetic and clinical remission. 13-cis-retinoic acid might have benefited this patient either by inducing differentiation of the malignant clone or by inhibiting its proliferation, allowing normal marrow elements to repopulate.

13-cis-retinoic acid has been shown to induce differentiation in cells from a patient with congenital neutropenia. Irreversible differentiation is also induced in cells of the promyelocytic leukemic cell line, HL-60, and in a minority of cells freshly obtained from patients with acute promyelocytic leukemia. In this patient, J.L., in vitro studies demonstrating complete inhibition of proliferation of the abnormal monocytoid clone by concentrations of 13-cis-retinoic acid similar to those he was achieving in vivo suggest that his clinical improvement was due to in vivo inhibition of proliferation of his abnormal clone.

This effect, however, seemed completely reversible when the 13-cis-retinoic acid was removed, either in vivo by discontinuing the drug or in vitro by washing the marrow cells free of the drug before placing them into culture. This reversibility has previously been noted in studies in many other systems in which 13-cis-retinoic acid either inhibits proliferation or prevents carcinogen- or radiation-induced tumor growth. It is likely that renewed clonal proliferation occurred while therapy was discontinued because several months later the same abnormal clone could again be demonstrated in his marrow cells. In vitro studies would have predicted this regrowth since clonal growth was inhibited in the presence of 13-cis-retinoic acid but was abundant in the presence of CSA or dexamethasone.

Continued inhibition of in vivo clonal proliferation by orally administered drug might have accounted for his initial complete remission. Similarly, the current return of his marrow cellularity to normal, the decrease in marrow blasts, the normalization of his platelet counts, and the return of normal granulocytes may be due to such inhibition.

It is of interest that the abnormal clone was monocytoid and that monocyte colony development from normal progenitor cells is inhibited by 13-cis-retinoic acid. Since 13-cis-retinoic acid is effective in only some cases of myelodysplastic syndrome, it may be that clinical responses occur in cases in which a monocytoid clone predominates. Studies of other patients with similar clinical courses and clonal histochemi-
cal and chromosomal markers will be required to evaluate this hypothesis further.

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