Retinoic Acid Treatment of Acute Promyelocytic Leukemia: In Vitro and In Vivo Observations

By P. J. Flynn, W. J. Miller, D. J. Weisdorf, D. C. Arthur, R. Brunning, and R. F. Branda

We describe in vitro studies and a therapeutic trial of retinoic acid (RA) in a patient with acute promyelocytic leukemia (APL) refractory to chemotherapy. Bone marrow promyelocytes from the patient, prior to RA, matured morphologically in liquid culture with RA (97% maturing myeloid cells compared with 26% in control cultures at 7 days). RA-cultured cells displayed leukocyte alkaline phosphatase activity and cytoplasmic maturation (by electron microscopy). Retinoic-acid-treated cells, compared to controls, demonstrated increased functional maturation, with phagocytosis of opsonized zymosan (90% versus 10%) and production of superoxide (measured by nitroblue tetrazolium reduction) in response to phorbol ester, opsonized zymosan, or the chemotaxin F-met-leu-phe. There was no evidence of active proliferation in the cultures. RA-treated cells continued to show 15:17 chromosomal translocation after 7 days in culture. The patient was treated with oral 13-cis-retinoic acid (100 mg/sq m/day) for 13 days. During that time, the peripheral white blood count rose from 300 cu mm to 6,700 cu mm, and the maturing myeloid cell count rose from 54 cu mm to 3,800 cu mm. Bone marrow maturing cells increased from 1.8% to 8.0%. Despite the increasing number of maturing myeloid cells, the patient died on day 13 from disseminated candidiasis. These data confirm that RA induces maturation of leukemic promyelocytes in vitro and suggest that similar maturation is achievable in vivo. We suggest that oral retinoic acid may be a useful adjunct in the treatment of APL.

ACUTE NONLYMPHOCYTIC LEUKEMIA (ANLL) is characterized by impaired maturation and differentiation of a clone of granulocyte precursors. Chemically induced cellular differentiation has been studied primarily in tumor cell lines, including the human promyelocytic leukemia line, HL-60. Polar-planar compounds, such as dimethyl sulfoxide (DMSO), induce granulocytic differentiation, while phorbol esters induce monocytic differentiation of HL-60 cells. While conventional chemotherapy for ANLL destroys immature cells, an alternative approach would be to induce maturation of these cells.

Observations that vitamin-A-deficient rats develop premalignant epidermal lesions that are reversible with repletion of retinoids first suggested that physiologic compounds may be used as differentiation inducers. In vitro studies confirmed that retinoic acid (RA), a vitamin A derivative, inhibits chemically induced malignant transformation and causes differentiation of HL-60 promyelocytes. Recent work has shown that RA induces maturation of cells from patients with acute promyelocytic leukemia (APL), while other morphological types of ANLL cells are unresponsive to retinoids.

The therapeutic usefulness of RA in leukemia is currently unproven, although beneficial effects are theoretically possible. The induction of functional maturation might offer the leukemic host protection from infections, thereby decreasing risk of a major complication. Clinical palliation might also result from terminal differentiation and reduced proliferative capacity of leukemic cells. RA may induce proliferative leukemic cells to mature, lose mitotic potential, and eventually die, resulting in reduced tumor load. Indeed, HL-60 cells cultured with RA show markedly diminished proliferative capacity. Whereas clinical trials of other maturation inducers have been deterred by toxicity considerations, 13-cis-retinoic acid has been used for a variety of dermatologic problems and appears safe. Levels sufficient to induce maturation of promyelocytes in vitro are obtainable in vivo without prohibitive side effects.

We describe in vitro culture studies and a therapeutic trial of 13-cis-RA in a patient with refractory APL. Bone marrow promyelocytes from this patient matured morphologically and functionally in culture with RA. This patient, whose disease was refractory to chemotherapy, was treated with oral 13-cis-RA for 2 wk, during which time peripheral blood maturing myeloid cells rose dramatically and the bone marrow showed an increase in mature elements. We suggest that such treatment may be a useful adjunct in some patients with APL.

CASE REPORT

The patient was a 22-yr-old male who presented with leukopenia in May 1980, and subsequently, acute promyelocytic leukemia was diagnosed by bone marrow. The bone marrow aspiration revealed 6% myeloblasts and 24.8% promyelocytes, many of which contained multiple Auer rods. Cytogenetic studies of bone marrow aspirate from the Departments of Medicine and Laboratory Medicine, University of Minnesota, Minneapolis, MN.

Supported in part by grants from the American Cancer Society (CH-220), and the National Institutes of Health (CA 28234). W.J.M. and D.J.W. are recipients of New Investigator Awards (R23-CA 32107 and AI 18160). R.F.B. is the recipient of a Research Career Development Award (CA 00657).

Submitted January 5, 1983; accepted July 11, 1983.

Address reprint requests to Dr. Wesley Miller, Box 37 Mayo Building, University of Minnesota Hospitals, 420 Delaware Street SE, Minneapolis, MN 55455.

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demonstrated a 15;17 chromosomal translocation. Initial therapy with daunomycin, cytosine arabinoside, and prednisone resulted in a maintained complete remission of 15 mo duration. In October 1981, neutrophil and platelet counts fell and bone marrow showed recurrent APL. Attempts at reinduction with daunomycin, cytosine arabinoside, and 6-thioguanine, and later with high-dose cytosine arabinoside plus m-AMSA, were unsuccessful. Four weeks after his last chemotherapy was finished, with no observed beneficial response, this study was initiated. At the time of this study, the patient was severely neutropenic and had a hypercellular (nearly 100% packed on biopsy) marrow consisting of (96%) blasts and abnormal promyelocytes (many with multiple Auer rods). The patient was continuously severely neutropenic (<96% neutrophils) and abnormal promyelocytes with condensation of nuclei in cells cultured with RA (Fig. 1). Electron micrographs confirmed the evidence of prominent azurophilic granules and a large open nuclear configuration in RA-cultured cells. Mitotic activity was assessed utilizing methyl-3H-thymidine (New England Nuclear, Boston, MA), with specific activity of 20 Ci/m mole for cellular incorporation. Two microCuries of thymidine was incubated with 2 x 10^6 cells at 37°C for 60 min, and then cell pellets were counted for radioactivity using a liquid scintillation counter.

Morphological Studies
Cultured cells were prepared for Wright-Giemsa staining by cytoospin. For each time point, three slides were reviewed with a differential of 200 cells counted per slide. Reported values are means of triplicate differentials. The following morphological criteria were used in identification of cell types. Promyelocytes demonstrated evidence of prominent azurophilic granules and a large open nuclear chromatin pattern with nucleoli. Myelocytes and metamyelocytes demonstrated loss of primary azurophilic granules, with development of secondary granules, but continued to have a large nucleus with open chromatin. Bands and segmented neutrophils (PMNs) demonstrated loss of primary azurophilic granules, development of secondary granules, and elongation or segmentation of condensed nuclei. Myelocytes, metamyelocytes, bands, and PMNs were considered to be maturing myeloid cells. Chloroacetate esterase, alpha-naphthyl acetate esterase, and leukocyte alkaline phosphatase stains were performed using standard techniques, and positively staining cells were enumerated.

Table 1. In Vitro Differentiation of Leukemic Promyelocytes: Effect of Retinoic Acid on Morphology

<table>
<thead>
<tr>
<th>Bone Marrow Sample</th>
<th>Days in Culture</th>
<th>Treatment</th>
<th>Promyelocytes</th>
<th>Myelocytes and Metamyelocytes (%)</th>
<th>Bands and PMNs (%)</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day pretreatment</td>
<td>7</td>
<td>Control</td>
<td>72</td>
<td>25</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Retinoic acid</td>
<td>1</td>
<td>81</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Day 8 on oral RA</td>
<td>8</td>
<td>Control</td>
<td>66.5</td>
<td>31</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Retinoic acid</td>
<td>0.5</td>
<td>63.5</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

Electron Microscopy
Specimens were prepared for electron microscopy by previously described methods.

Cytogenetics
Direct preparations of cultured cells were examined for cytogenetic abnormalities as previously described.

Functional Studies
Phagocytosis was assessed by counting the number of cells completely ingesting zymosan particles (500 μg/ml, 20 min, 37°C) that had been previously opsonized with fresh serum (30 min, 37°C). Oxygen radical generation was measured by reduction of nitroblue tetrazolium (NBT). Cells at 10^6/ml were incubated with 10 μg/ml NBT (20 min, 37°C). Cytospin Wright-Giemsa preparations were then counted to determine the number of cells containing reduced blue-black formazan deposits. NBT reduction was measured in unstimulated cells, as well as in those stimulated by opsonized zymosan particles, phorbol myristate acetate (100 ng/ml), and the synthetic chemotactic peptide, F-met-leu-phe (10^-7 M). NBT, leukocyte alkaline phosphatase stain, and phorbol were obtained from Sigma Chemical Co., St. Louis, MO. Zymosan was obtained from ICN Nutritional Biochemicals, Cleveland, OH. F-met-leu-phe was obtained from Calbiochem, La Jolla, CA.

Treatment of the Patient
The patient signed written consent for administration of 13-cis-retinoic acid (a gift from Hoffmann-LaRoche Inc., Nutley, NJ), and these studies were performed after approval by the Committee for the Use of Human Subjects in Research. Twenty-eight days after his last chemotherapy, the patient was begun on oral 13-cis-retinoic acid at 100 mg/sq m/day, with a planned treatment course of 2 mo. At the initiation of treatment, the patient was severely neutropenic, with a hypercellular bone marrow.

RESULTS
In Vitro Studies
Bone marrow aspirates were obtained 1 day prior to and after 8 days of oral retinoic acid. Liquid cultures from both samples demonstrated morphological maturation in presence of RA (Table 1). The RA-cultured cells from the pretreatment marrow differentiated to 16% bands and PMNs. RA cultures from the day-8 marrow produced 36% bands and PMNs, suggesting that, after oral RA, leukemic cells underwent more complete differentiation in vitro. Light microscopic examination of RA-cultured cells showed less prominent primary granules and development of secondary granules with condensation of nuclei in cells cultured with RA (Fig. 1). Electron micrographs confirmed the
Fig. 1. Control cells (A, C) demonstrate, by light microscopy (A), prominent azurophilic granules, fine nuclear chromatin pattern, prominent nucleoli, and high nuclear/cytoplasmic ratio. On ultrastructural examination (C), a prominent feature of many of the cells is marked dilatation of cisternae of endoplasmic reticulum. Scattered azurophilic granules are present, and the nuclear chromatin is finely dispersed. RA-treated cells (B, D), by light microscopy (B), show loss of primary and appearance of secondary granulation, nuclear condensation and segmentation, and reduced nuclear/cytoplasmic ratio. On ultrastructural examination (D), the majority of cells contain abundant azurophil granules and small rounded profiles of endoplasmic reticulum. Nucleoli are usually readily identified and are occasionally prominent. There is a prominent golgi in one of the cells. (Light microscopy, Wright’s stain, original magnification ×640; electron microscopy, uranyl acetate-lead citrate, original magnification ×14,400). Figs. 1C and D on p. 1214.
Fig. 1 (cont'd.).
cytoplasmic maturation, although the nucleoplasm remained immature (Fig. 1).

Further studies to characterize the pretreatment bone marrow aspirates after 7 days in liquid culture demonstrated that leukocyte alkaline phosphatase activity was positive in 20% of RA-treated cells, while control cells were all negative. Chloroacetate esterase activity was present (>95%) in control and RA-treated cells, while alpha-naphthyl acetate esterase stain remained negative in both groups. These histochemical stains confirmed that the RA-treated promyelocytes underwent granulocytic (rather than monocytic) differentiation.

Pretreatment leukemic cells after 7 days in culture showed a marked increase of functional maturity with RA, but not in controls (Table 2). Most cells (>80%) cultured with RA produced superoxide (measured by NBT reduction) in response to chemotactic and phagocytic stimuli as well as in response to phorbol myristate acetate. Additionally, morphological studies confirmed phagocytic capability in 90% of cells cultured with RA.

Assays on culture day 7 revealed that cells with media alone, ethanol, or retinoic-acid-ethanol were greater than 90% viable by trypan blue exclusion. Cell counts were nearly identical in all cultures at 7 days and showed only a slight increase over cell counts at the beginning of the incubations: $7.0 \times 10^5$ cells with RA, $6.0 \times 10^5$/ml with ethanol, and $6.5 \times 10^5$/ml with media alone. Furthermore, cultured cells ($2 \times 10^6$ cells) showed no evidence of mitotic activity by $^3$H-thymidine incorporation (background counts $23.6 \pm 11.4$ cpm, control cells $14.7 \pm 0.3$ cpm, RA-treated cells $19.2 \pm 1.6$ cpm). The lack of proliferation and mitotic activity suggested that the maturing cells in RA-treated cultures originated from malignant promyelocyte maturation rather than from replicating normal or leukemic cell populations. In addition, at day 7 of culture with RA, 3 of 4 evaluable metaphases continued to show the 15;17 chromosomal translocation that characterized this patient's leukemia, confirming continued presence of the leukemic cells.

Clinical Studies

The patient was treated for 13 days with oral cis-retinoic acid, and the medication was reasonably well tolerated. Worsening of preexisting stomatitis was noted and may have been related to toxicity. Three days after the initiation of therapy, both the total white count and the maturing myeloid cells (as defined in Materials and Methods) began to rise, and over a 2-wk period, reached highs of 6,700/cu mm and 3,800/cu mm, respectively (Fig. 2). The differential of peripheral blood was myeloblasts and promyelocytes—40%, myelocytes and metamyelocytes—43%, bands and PMNs—15%, and lymphocytes—2%. The morphology of the maturing myeloid cells (Fig. 3) closely resembled that seen in RA-treated cultures (Fig. 1). Bone marrow examination immediately prior to instituting retinoic acid therapy showed 96% myeloblasts and promyelocytes plus 1.8% maturing myeloid cells (myelocytes and metamyelocytes—1.0%; bands and PMNs—0.8%) in a hypercellular biopsy. A bone marrow on day 8 of therapy showed 95% myeloblasts and

![Fig. 2. Appearance of circulating mature leukocytes in a patient with APL during treatment with retinoic acid (RA).](image-url)
promyelocytes, plus 2.0% maturing myeloid cells (myelocytes and metamyelocytes—1.0%; bands and PMNs—1.0%), while a repeat marrow on day 13 showed 87.2% myeloblasts and promyelocytes plus 8.0% maturing myeloid cells (myelocytes and metamyelocytes—5.0%; bands and PMNs—3.0%), suggesting that the rise in peripheral white blood count reflected maturation of cells in the bone marrow. Despite the encouraging rise in leukocytes, the patient succumbed and at autopsy was found to have a disseminated Candida infection.

**DISCUSSION**

Cytotoxic chemotherapy induces remission in the majority of patients with ANLL. The need for additional modes of therapy is underscored by the high incidence of relapse, with eventual resistance to chemotherapy among such patients. Our studies are encouraging in that a patient with refractory APL, when treated with oral cis-retinoic acid, showed a dramatic rise in peripheral blood maturing myeloid cells. These maturing myeloid cells were morphologically similar to cells observed when bone marrow promyelocytes were cultured in vitro with RA. Cells from RA-containing cultures developed functional maturity with increased capability for superoxide production and phagocytosis. Cell maturation with RA was confirmed by demonstration of leukocyte alkaline phosphatase (LAP) activity. Despite evidence of maturation, it must be emphasized that these maturing cells were not morphologically normal granulocytes. Nuclei remained immature on electron microscopy, and the cells showed continued cytogenetic abnormality.

Although the cells matured functionally in vitro, RA-induced maturation in vivo did not protect the host from death due to disseminated candidiasis.

The role of retinoic acid in treatment of APL is presently unclear. Clinical palliation may result through terminal differentiation and reduced proliferative capacity of leukemic cells. Cultured cells from our patient showed no evidence of mitosis in culture with or without RA, and we are therefore uncertain of the effect of RA on proliferation of his cells. However, HL-60 cells, which grow well in culture, show a marked decrease in cell division with RA, suggesting that RA-induced terminal differentiation and slowed cell growth may be a feasible approach to reduction of tumor load. Although unproven as a remission induction therapy without cytotoxic chemotherapy, RA may be an effective adjunct by inducing differentiation and reducing proliferation of leukemic cells. Further studies are needed to define the therapeutic usefulness of retinoic acid in acute promyelocytic leukemia.

**ACKNOWLEDGMENT**

We wish to thank Dr. Jack Alexander for care of the patient, and Carol Taubert for assistance in manuscript preparation.

**REFERENCES**


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