Resistance to All-Trans Retinoic Acid (ATRA) Therapy in Relapsing Acute Promyelocytic Leukemia: Study of In Vitro ATRA Sensitivity and Cellular Retinoic Acid Binding Protein Levels in Leukemic Cells

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Since 1988 all-trans retinoic acid (ATRA) has been proposed as an alternative therapy for acute promyelocytic leukemia (APL), a specific acute myeloid leukemia (AML) subtype characterized by a chromosomal translocation t(15;17) involving the retinoic acid receptor α (RARα) gene on chromosome 17 and the promyelocytic (PML) gene on chromosome 15.4,10 We and others have shown that a high proportion of patients with AML3 achieve complete remission (CR) with oral ATRA alone.11-15 This response is obtained via a novel differentiation mechanism, now well documented by morphologic,12 immunophenotyping,14 in situ hybridization,14 and DNA polymorphism.15,16 studies of the leukemic maturing clone.

In normal cells, RA mediates its biologic effect via six individual nuclear receptors encoded by distinct genes (RARs, RXRs). Specific RA isomers, and, in some cases, cellular RA-binding proteins, CRABPI and II. CRABPs are efficiently activated by ATRA and 9-cis RA, whereas RXRs, at ligand concentrations, are only activated by 9-cis RA. Interestingly, RARs and RXRs bind more effectively to specific RA-response elements as heterodimers (RAR-RXR). In the cytoplasm, RA binds to CRABPs. CRABP's function is under extensive study; it appears that it should facilitate the transport of RA to the endoplasmic reticulum, where it is metabolized by degradative enzymes.26-29 The mechanism of action that underlies the exquisite sensitivity of AML3 cells and AML3 patients to ATRA is not yet clear. In addition, AML3 cells have an abnormal RA-binding protein (PML/RARα) resulting from the t(15;17) translocation. One may surmise that ATRA can mediate differentiation in AML3 cells via the PML/RARα protein and/or the normal RARs in a dose-dependent manner. Furthermore, no CRABPII protein has been detected in AML3 cells before any ATRA therapy as in HL-60 and U-937 cells, its role in ATRA-mediated differentiation in these cells is not yet settled.

Unfortunately, experience has shown that the duration of the remissions maintained with continuous ATRA therapy alone are short, and, when relapse occurs shortly after ATRA withdrawal, the ATRA fails to induce a second remission. Putative mechanisms for this in vivo resistance have included additional chromosomal abnormalities, selection of non-AML3 leukemic clones, mutations in the RARs gene as noted in HL-60 cells,33 continuous ATRA treatment in cell lines,34-36 or interference by increased expression of proteins involved in ATRA's metabolism.28,37

To investigate what features of the AML3 cells at relapse could explain the in vivo resistance observed, we studied the CRABP levels and in vitro sensitivity to ATRA of AML3 cells before and at relapse from ATRA therapy. Relapse-AML3 cells (n = 12) showed reduced differentiation induction when compared with "virgin"-AML3 cells (n = 31; P < .05). Dose-response studies were performed in 2 cases at relapse and showed decreased sensitivity to low ATRA concentrations. CRABPII levels and in vitro differentiation characteristics of AML3 cells before and at relapse from ATRA therapy were studied concomitantly in 4 patients. High levels of CRABPII (median, 20 fmol/mg of protein) were detected in the cells of the 4 patients at relapse but were not detected before ATRA therapy. These three of these patients showed a decrease in differentiation induction of their leukemic cells, and a failure to achieve CR with a second induction therapy of ATRA 45 mg/m²/day was noted in all patients treated (n = 3). Results from this study provide evidence to support the hypothesis of induced-ATRA metabolism as one of the major mechanisms responsible for ATRA resistance. Monitoring CRABPII levels after ATRA withdrawal may help to determine when to administer ATRA in the maintenance or relapse therapy of AML3 patients.

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Blood, Vol 82, No 7 (October 1), 1993: pp 2175-2181

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Submitted February 24, 1993; accepted June 17, 1993.

Supported in part by grants from l’Association pour la Recherche contre le Cancer, la Fondation de France contre la Leucémie, and la Ligue contre le Cancer.

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0006-4971/93/8207-0027$00.00/0
induced during ATRA therapy and result in overall insufficient nuclear-ATRA levels that lead to reduced RA-sensitivity in vivo.

MATERIALS AND METHODS

Patients. During the period September 1990 to September 1991, 12 AML3 patients with the specific translocation t(15;17) were studied at relapse after ATRA-induced CR. First induction ATRA therapy (45 mg/m²/day) was administered to these patients until CR was obtained (median, 3 months). After achievement of CR, the patients were given low-dose maintenance chemotherapy (6-mercaptopurine/methotrexate n = 11; cytarabine/methyl GAG n = 1), and 1 patient was kept on ATRA alone. Relapse occurred after a median withdrawal interval from ATRA of 6 months (0 to 26 months) (Table 1). Six patients were treated with a second ATRA induction therapy (45 mg/m²/d). Failure, as noted by an absence of in vivo cytologic signs of maturation in the blood and bone marrow (BM) and a rapid increase of blast cells leading to the addition of conventional chemotherapy, was followed by early death in 5 cases after a median therapy of 15 days.

Cell samples and culture. BM samples were collected after informed consent in heparinized tubes before the first ATRA therapy and when relapse occurred. Leukemic cells were isolated after adherence of monocytes to the plastic surface. Leukemic cells were cultured in RPMI 1640 (GIBCO, Paisley, UK) supplemented with 15% fetal calf serum (FCS; Seromed, Berlin, Germany), L-glutamine (2 mmol/L), penicillin (100 IU/mL), and streptomycin (1%; GIBCO), incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, and subcultured twice a week. Cell number and viability were estimated by trypan-blue exclusion dye test.

The enriched leukemic cell fraction (>90% blasts) was suspended in culture medium at a concentration of 10⁶ cells/mL in the presence of ATRA (provided by Dr W. Bollag, Hoffmann-La Roche, Basel, Switzerland). Cell aliquots were taken at days 3 and 5 or later for assessment of viability and differentiation status. Differentiation was assessed on morphologic and functional criteria. Slides were stained with May-Grünewald-Giemsa stain (Laboratoire Central d’Hématologie, Hôpital Saint-Louis). The nitroblue tetrazolium (NBT) reduction test was performed using standard methodology with phorbol myristate acetate (PMA) as previously described. Differential counts were performed under light microscopy on a minimum of 200 cells. The percentage of cells containing intracellular-reduced black formazan deposits was thus determined.

Preparation of cytosolic extracts. Fresh human leukemic cells (30 × 10⁶) were washed once in medium without FCS and in cold sodium phosphate buffer before protein extraction. Cells were homogenized at 4°C in 500 μL of an ice-cold extraction buffer (Tris, 100 mmol/L; NaCl, 50 mmol/L; EDTA, 5 mmol/L; dithiothreitol, 2 mmol/L; pH 7.4) with three 30-second strokes of a Polytron (Kinematica; GiMbh, Luzern, Switzerland) at full speed. Supernatants were obtained by centrifugation at 100,000g at 4°C for 60 minutes, distributed in 100 μL aliquots, and frozen at −20°C or used immediately.

Incubation of cytosolic extracts with ATRA. All manipulations were performed under dim or yellow light. Supernatant aliquots of 100 μL containing 300 μg protein were incubated with 300 nmol/L of [³H]-ATRA (specific activity, 2,035 GBq/mmol) from DuPont New England Nuclear (Boston, MA) at 4°C for 16 hours. For binding specificity a 200-fold excess of unlabeled ATRA (Sigma Chemical Co, St Louis, MO) was added in the same manner as for the radioactive compounds.

Slab polyacrylamide gel electrophoresis (PAGE). At the end of the incubation period, an aliquot of the supernatant (70 μL) was subjected to vertical slab PAGE under dim light at 14°C as described by Siegenthaler et al with slight modifications of the method. The proteins were separated according to their different electrophoretic mobilities in the gel, as a consequence of their net charge. This technique allows the separation of CRABPI and II. The bands were collected and treated overnight with 400 μL Protocol (DuPont, Boston, MA) in 5-mL scintillation vials before 4 mL of solubilization cocktail was added. The radioactivity was then determined in a scintillation counter.

Statistical analysis. Differences in the percentage of differentiated cells at onset and at relapse were assessed by the parametric Sign test.

RESULTS

In vitro differentiation of AML3 cells from patients relapsing from ATRA therapy. In vitro response to ATRA 10⁻⁶ mol/L was studied in 12 AML3 patients who relapsed after ATRA therapy (45 mg/m²/d). Table I summarizes the clini-

<table>
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<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>Length of First Induction ATRA Therapy (mo)</th>
<th>Disease Free Survival After ATRA Withdrawal (mo)</th>
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<td>NT</td>
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Abbreviation: NT, not treated with ATRA.
cal characteristics of these patients before relapse occurred, namely length of ATRA therapy and of the disease-free survival under low-dose maintenance chemotherapy. All patients at relapse after ATRA therapy had a cytologic and/or cytogenetic diagnosis of AML3.

The purified leukemic cells were cultured for 5 days in the presence of ATRA $10^{-6}$ mol/L. Compared with 31 virgin AML3 cell samples studied during the same period, relapse cases achieved a lower percentage of differentiated cells (median 46% vs 90% NBT positive cells, $P < .05$; Fig 1A). Eight of the 12 relapse cases (case nos. 1 through 7 and 11) and only 4 of 31 virgin cases showed less than 70% differentiated cells using the oxidative burst function criteria (Fig 1A). Three of the relapse cases had less than 35% differentiated cells (case nos. 2, 3, and 5), and one case did not respond (case no. 1). Absence of morphologic modifications correlated with the poor functional achievement. Cell count was similar in these groups (med $1 \times 10^6$ cells/mL in virgin AML3 samples vs $0.8 \times 10^6$ cells/mL at relapse). Therefore, the failure of differentiation could not be due to an increased proliferation of the undifferentiated AML3 cells. Virgin AML3 cells are extremely sensitive to ATRA, with 80% of the cell population already reducing NBT at $10^{-7}$ mol/L and 45% at $10^{-8}$ mol/L (Fig 1A and B). Sensitivity to low ATRA concentrations was studied in 2 AML3 samples at relapse after ATRA therapy (case nos. 2 and 10). In both cases, absence of sensitivity to ATRA concentrations of $10^{-7}$ mol/L and $10^{-8}$ mol/L was observed (Fig 1B).

The in vitro response to ATRA $10^{-6}$ mol/L of 12 AML3 patients at relapse was compared with the differentiation achieved on the AML3 cells of these same 12 patients before any ATRA therapy. A decrease in the percentage of differentiated cells in the leukemic cell culture was clear in half of the cases ($P < .03$; 5 cases [nos. 1 through 5] with a reduction of at least 23% at $10^{-6}$ mol/L of ATRA; Fig 2A). Low dose-response analysis was possible in 1 patient (case no. 10) before ATRA therapy and when relapse occurred. A shift to the right of the dose-response curve at relapse was observed (Fig 2B). These data suggest that a decrease in ATRA sensitivity may not be detected in all AML3 cell samples at relapse with a saturating $1 \mu$mol/L concentration of ATRA and that lower doses should be tested at onset and at relapse.

In 4 cases (nos. 2, 4, 5, and 10), higher ATRA concentrations were studied to test the hypothesis that relapse-AML3 cells could be triggered to differentiate with higher ATRA concentrations. Effective restoration of differentiation was only observed in 1 case (no. 2) after incubation with $10^{-5}$ mol/L ATRA. Cases no. 4 and 10 already had a relatively good response at $10^{-6}$ mol/L and case no. 5, which had a poor response, was not improved by higher RA concentrations (Table 2).

Presence of CRABPII in relapse-AML3 cells. In a previous study, we reported the induction of the CRABPII in normal hematopoietic cells of AML3 patients undergoing ATRA therapy (Figs 3A and B; and Figs 4A, B-1, and B-2). CRABPII is distinguished from CRABPI by a different binding affinity for retinoids and a different isoelectric point detected by PAGE. Cytosol extracts of 4 AML3-
Fig 2. (A) In vitro differentiation of AML3 cells of 12 patients with ATRA 10^-6 mol/L before and after ATRA therapy at relapse (expressed as the percentage of NBT positive cells at relapse minus the percentage at onset of ATRA therapy). (B) Dose-effect of ATRA on patient no. 10 AML3 cells at onset (C) and at relapse after ATRA therapy (W).

Table 2. Dose-Effect of ATRA Differentiation in Relapse-AML3 Cells

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<th>Patient No.</th>
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<th>10^-6 mol/L</th>
<th>Control</th>
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</table>

Values are the percentage of differentiated cells after 5 days of in vitro culture with ATRA.

Abbreviation: ND, not determined.
Two explanations may be forwarded for this apparent discrepancy between in vitro and in vivo response at relapse. The first is that the concentration of ATRA prevailing in most in vitro differentiation assays (10−7 mol/L) is above the maximal effective concentration in the AML3 system. We have previously stressed the potency of a 10−7 mol/L concentration to induce maximal differentiation in AML3 cells. A decrease in ATRA-induced differentiation might have been observed in all relapse cases if nonsaturating concentrations (10−7 mol/L) had been used. In fact, in the only 2 cases in which this could be tested, we were able to show a clear shift to the right of the dose-response curve at low ATRA concentrations, suggesting that AML3 cells at relapse may well have a reduced sensitivity to ATRA. To study this hypothesis, in vitro models are being set up in the laboratory.

The second explanation is that poor in vivo response may not necessarily be attributed to the characteristics of the relapsing leukemic cell alone. Low ATRA plasma levels have been reported during ATRA therapy. We have previously linked low plasma ATRA concentrations to increased clearance rate and poor in vivo response. An overall increase in ATRA catabolism in cell tissues by an increase of CRABP, as it has been shown in the skin and hematopoietic tissue, may account for the low plasma ATRA concentrations observed during continuous ATRA therapy and for failure to achieve effective ATRA intracellular concentrations.

Thus, one may hypothesize that, in AML3 patients, ATRA therapy induces a cascade of events. The aim of one of these is to subsequently reduce the intracellular concentration of ATRA. Increase in the level of proteins linked to ATRA’s metabolism during ATRA therapy is one major step in this process. Induction of CRABP1I in AML3 patients may result from a direct regulation by ATRA, because an RA-responsive element has been reported in the promoter region of the mCRABP1I gene. Along with the increase of CRABP, increased levels of cytochrome P450 enzymes have been observed in ATRA-treated hamsters and linked to the hypercatabolistic state observed in AML3 patients treated with ATRA. For some of these patients, we have different data showing that, at one point during CR, CRABP levels were no longer detectable. This suggests that a residual leukemic clone that was CRABP positive during ATRA therapy persisted at CR. Furthermore, the fact that high levels of CRABP are detected both in the normal and leukemic BM cells of AML3 patients during ATRA therapy suggests that the hypercatabolistic state may be responsible both for the relapses observed if ATRA is maintained as consolidation therapy and for the subsequent resistance to ATRA when relapse occurs.

The majority of the patients in this early clinical trial (1990) were either still under ATRA therapy or after a short withdrawal period from ATRA when they relapsed, forwarding an explanation for the presence of CRABP1I in the leukemic cells. CRABP1I levels decrease slowly (median, 1 month) after ATRA withdrawal. Therefore, it may be expected that the hypercatabolistic state may eventually be reduced after ATRA withdrawal and sensitivity to ATRA restored. In fact, 2 relapse-AML3 patients have been recently shown to respond to a second ATRA therapy, after withdrawal period of 6 months and 2 years (S. Castaigne, and R.P. Warrell Jr, personal communications, 1992). Although one of the patients in this study relapsed and failed to respond to ATRA after a withdrawal period of 10 months, one must keep in mind that most of the patients in this study had already relapsed after previous chemotherapy before receiving their first induction ATRA therapy and may have had other factors leading to reduced RA sensitivity in vitro and in vivo. It is equally important to stress that, although the increased ATRA metabolism may be acquired in all AML3 patients undergoing ATRA therapy, alternative or coexisting factors of resistance, such as molecular changes and selection of non-AML3 clones, cannot be ex-
cloned and have occasionally been observed (C.C., personal observations, 1993).

Recent results42 from our study group suggest that maintenance therapy in AML3 could be improved if ATRA was combined with chemotherapy. Thus, it is necessary to overcome the inconvenience of induced RA resistance. Strategies to decrease the induction of the hypercatabolic state (lower ATRA concentrations, shorter induction regimens, choice of new retinoids, or metabolizing, limiting drugs such as ketokonazole43) are desirable. Monitoring CRABP levels by PAGE or reverse transcriptase-polymerase chain reaction (undergoing study) after ATRA withdrawal may determine the best time and schedule to readminister ATRA in the maintenance or relapse therapy of AML3 patients.

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Resistance to all-trans retinoic acid (ATRA) therapy in relapsing acute promyelocytic leukemia: study of in vitro ATRA sensitivity and cellular retinoic acid binding protein levels in leukemic cells [see comments]

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