Continuous Treatment With All-Trans Retinoic Acid Causes a Progressive Reduction in Plasma Drug Concentrations: Implications for Relapse and Retinoid “Resistance” in Patients With Acute Promyelocytic Leukemia


Although all-trans retinoic acid (RA) induces complete remission in a high proportion of patients with acute promyelocytic leukemia (APL), all groups have described clinical relapses despite continued RA treatment. This finding suggests that resistance to the cytodifferentiating effects of the retinoid had been acquired. To investigate potential mechanisms of clinical resistance to RA, we serially evaluated the clinical pharmacology of the drug in APL patients treated with this agent. Leukemic cells from patients relapsing from RA treatment were cultured in the presence of RA and examined for evidence of morphologic maturation. We also studied messenger RNA expression of the newly described gene product of the (15;17) translocation in APL, PML/RA receptor-α (PML/RAR-α). Serial pharmacokinetic studies showed that continuous daily RA treatment was associated with a marked decrease in plasma drug concentrations at the time of relapse compared with the initial day of therapy.

Recent studies have shown that a high proportion of patients with acute promyelocytic leukemia (APL) achieve complete remission after treatment with all-trans retinoic acid (RA).1,2 However, the duration of these remissions has generally been brief, and all groups have reported relapse despite continuous RA treatment.3-5 These results have suggested that resistance to the antileukemic effects of RA was acquired during drug therapy. Several mechanisms of acquired retinoid resistance have now been characterized in cell lines. Putative mechanisms of in vitro resistance have included new mutations in nuclear retinoid receptors6,7 or alterations in the absolute amount or binding affinity of cellular RA binding proteins (CRABP).8,9

To investigate the mechanisms of clinical retinoid resistance, we evaluated patients who received RA as induction treatment for APL with serial pharmacokinetic studies on the first day of treatment, at the time of relapse, and again after a twofold RA dose escalation. Leukemic cells obtained from four patients who relapsed during RA treatment were cultured in vitro with RA and were examined for morphologic evidence of differentiation. In two relapsing patients, messenger RNA (mRNA) expression of the newly described fusion gene product of the (15:17) translocation in APL, PML/RA receptor-α (PML/RAR-α),8,9 was also studied to explore whether further abnormalities had been induced in this receptor during RA treatment. Results from these studies suggest that clinical resistance to RA treatment in APL may result from an induced pharmacologic depression of plasma drug concentrations, potentially to levels that are inadequate to sustain differentiation in vivo.

Materials and Methods

Clinical information. Patients in this study were undergoing remission induction treatment for APL with all-trans RA at a dose of 45 mg/m²/d as recently described.3 Six patients maintained sole on RA relapsed during treatment and had no further clinical response despite an increase in the daily dose from 45 to 90 mg/m²/d.

Pharmacokinetic studies. On day 1 of therapy, patients received a single oral RA dose of 45 mg/m². Heparinized blood samples were obtained before drug administration and at 0.5, 1, 2, 3, 4, 6, and 24 hours. Urine was collected in three aliquots from 0 to 6, 6 to 12, and 12 to 24 hour intervals after drug ingestion. These assays were repeated at the time of relapse in six patients who were maintained on the drug after having achieved complete remission (CR). At that time, the pharmacokinetic study was performed on the final day of treatment with the dose used for maintenance (45 mg/m²/d). Doubling the RA dose in six patients failed to reinduce response at the time of relapse and also failed to significantly augment plasma RA concentrations. However, leukemic cells obtained at the time of relapse from four patients retained in vitro sensitivity to the differentiating activity of RA (10⁻⁴ mol/L). No change was observed in the pattern of PML/RAR-α expression assessed by Northern blot analysis at the time of relapse compared with pretreatment in two patients who were tested. These results indicate that clinical relapse and “resistance” to continuous treatment with all-trans RA in APL is associated with progressive reduction of plasma concentrations, potentially to levels below those that sustain differentiation of leukemic cells in vivo. Long-term success of this treatment will require the development of strategies that circumvent this pharmacologic phenomenon.

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mg/m²/d) and again shortly after the RA dose was doubled to 90 mg/m²/d.

Reagents and analytical techniques. All-trans-9-nosityl-3,7-

•dimethyl-2,4,6,8 nonatetraenoic acid (the internal standard), 4-oxo-

•13-cis RA, and 4-oxo-all-trans RA were kindly supplied by Dr F. Vane (Hoffmann-LaRoche, Nutley, NJ). β-Gluconidase type VII-A (from Escherichia coli), all-trans RA, and 13-cis RA were purchased from Sigma Chemical Co (St Louis, MO). Other chemicals were from either Baker Chemical Co (Phillipsburg, NJ) or Fisher Scientific (Fair Lawn, NJ) and were of HPLC or highest grade available. Because retinoids may degrade with exposure to light, samples were transported in amber-colored plastic bags and were processed under low-light conditions.

Plasma samples were extracted and assayed by HPLC as described by Bugge et al. Retinoids were detected by UV absorption at 365 nm (attenuation = 0.01). Selected plasma samples that fell below the sensitivity limits of this HPLC technique (10 ng/mL) were assayed by a recently described liquid chromatography/mass spectrometry method. Standard curves for all-trans RA, 13-cis RA, and their 4-oxo metabolites were established using pooled normal plasma and were linear over a range from 0 to 1,000 ng/mL. The plasma half-life was estimated using computerized software (PCNONLIN84; Statistical Consultants, Inc, Lexington, KY), and the area under the concentration × time curve (AUC) was determined by trapezoidal approximation.

Leukemia cell cultures. Heparinized bone marrow aspirates were obtained from four patients at presentation and again at the time of relapse from RA treatment. The low density fraction of bone marrow mononuclear cells obtained after Ficoll-Hypaque centrifugation was washed twice with cold RPMI media and suspended in liquid cultures with media containing 10% heat-inactivated fetal calf serum and 1% glutamine. The cell suspensions were cocultured with all-trans RA (10⁻⁴ mol/L) (Hoffmann-LaRoche Inc) dissolved in 95% ethanol and grown in a 5% CO₂ humidified incubator at 37°C.

Leukemic cells from four patients who relapsed from CR while maintained solely on RA were cultured in vitro with RA (10⁻⁴ mol/L). Compared with controls, morphologic examination of RA-cultured cells showed changes consistent with maturation (ie, increase in nuclear chromatin condensation and partial nuclear segmentation), suggesting that leukemic cells from these clinically resistant patients retained in vitro sensitivity to the cytodifferentiating activity of RA.

Mean values, three individual patients were studied at all three time points. Mean plasma RA concentrations versus time are depicted in Fig 1; these intrapatient data show a similar pattern of plasma level reduction and lack of increase with increased dosage.

Of interest, we studied one patient who has remained in CR for the longest period in our study while maintained on continuous RA treatment. As shown in Fig 2, when this patient was studied while in CR at 8 months, her calculated plasma AUC was similar to her day 1 value (365 ± 406 ng·h/mL).

In vitro cellular differentiation. Leukemic cells from four patients who relapsed from CR while maintained solely on RA were cultured in vitro with RA (10⁻⁴ mol/L). Compared with controls, morphologic examination of RA-cultured cells showed changes consistent with maturation (ie, increase in nuclear chromatin condensation and partial nuclear segmentation), suggesting that leukemic cells from these clinically resistant patients retained in vitro sensitivity to the cytodifferentiating activity of RA.

RESULTS

Pharmacokinetics. Ten patients were evaluated on day 1 after treatment with a single oral RA dose of 45 mg/m². Oral administration of the first dose of RA produced peak plasma concentrations within 1 to 2 hours after administration. The calculated mean plasma AUC for a group of 10 patients on day 1 was 499 ± 200 ng · h/mL. Six patients were studied at the time of relapse; the mean AUC of these patients was significantly lower compared with the group mean value on day 1 (244 ± 145 ng · h/mL; P < .03). These six individuals then received 90 mg/m² orally; however, despite the twofold dose increase, their mean plasma AUC was not augmented (222 ± 112 ng · h/mL; P = .56) (Table 1). While the preceding data are comparisons of group

Table 1. Changes in Pharmacokinetic Parameters on Day 1 of Treatment Compared With the Time of Relapse After Continuous Daily Administration of All-Trans RA

<table>
<thead>
<tr>
<th>Time</th>
<th>Dose (mg/m²)</th>
<th>N</th>
<th>Mean AUC (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Day 1</td>
<td>45</td>
<td>10</td>
<td>499 ± 200</td>
</tr>
<tr>
<td>B. Relapse</td>
<td>45</td>
<td>6</td>
<td>244 ± 145</td>
</tr>
<tr>
<td>C. Relapse</td>
<td>90</td>
<td>6</td>
<td>222 ± 112</td>
</tr>
</tbody>
</table>

A v B, P = .03; B v C, P = .56; paired t-test.

Fig 1. Plasma concentrations (±SEM) of all-trans RA after the administration of a single oral dose (45 mg/m²) on day 1 of treatment in three patients (●), at the time of relapse (△), and at relapse (●) after treatment with a single dose of 90 mg/m². The calculated plasma AUC concentrations were significantly lower at the time of relapse compared with day 1 and were not increased despite the twofold escalation in dose.
We had originally surmised that continuous RA treatment during CR after the leukemic cell burden had been reduced might cause a progressive increase in plasma drug concentrations, thereby increasing toxicity over time. Unexpectedly, we found that continuous treatment was associated with progressively diminishing plasma levels. The onset of this effect is relatively rapid and occurs within 2–6 weeks from the initiation of treatment in most patients. This effect has not been previously described in human subjects, and it does not occur during therapy with other retinoids such as 13-cis RA or etretinate. However, our observations are consistent with recent data derived from other species. Creech Kraft et al. described a reduction in plasma levels of all-trans RA in two Cynomolgus monkeys that underwent serial studies, and Kalin et al. showed that RA pretreatment in mice reduced subsequent plasma AUC concentrations. Notably, treatment with 13-cis RA did not induce these effects in either species.

Several explanations could account for the pharmacologic behavior observed here. First, because biliary excretion accounts for at least 60% of RA elimination in rodents, it is conceivable that clinically unapparent malab-

**Northern blot analysis of PML/RAR-α** Serial Northern blot analysis of total cellular RNA from bone marrow mononuclear cells taken from two relapsing patients showed that there was no change in the migration of mRNA transcripts of PML/RAR-α or normal RAR-α before RA treatment or after relapse. Gels from one of these patients are illustrated in Fig 3. In these patients, an identical pattern of reverse transcriptase/polymerase chain reaction amplification products was also observed using specific primers for the fusion receptor molecule (Dr W.H. Miller Jr, unpublished data).

**DISCUSSION**

Despite a high initial CR rate, unexplained relapses have occurred in many patients with APL despite continued treatment with all-trans RA. This finding suggests that resistance to the cytodifferentiating actions of the retinoid had been acquired during the course of therapy; however, the mechanisms that underlie such resistance have not been previously examined in the clinical setting. Prior studies in vitro have shown that retinoid resistance can be induced by continuous culture in high concentrations of RA, and that this resistance is associated with acquired mutations in retinoic acid receptors or with alterations in the expression of CRABP. However, data from our study indicate that clinical resistance to all-trans RA therapy in APL may have a simpler pharmacologic explanation.
Absorption or nonspecific binding to intestinal proteins reduced the oral bioavailability. However, we previously noted that urinary excretion of the 4-oxo-all-trans RA metabolite increased approximately 10-fold with continuous daily treatment despite a decrease in peak plasma levels. Although this amount accounts for less than 1% of the administered dose in humans, these data and the absence of any other evidence for malabsorption suggest that an induced progressive impairment of gastrointestinal uptake is unlikely.

Second, continued RA treatment could result in further RAR-α mutations. Such mutations have been observed after F9 teratocarcinoma cells were rendered resistant after long-term cultures with high RA concentration in vitro. However, we failed to detect changes in the migration of the normal or mutated RAR-α species in either of our patients who were examined by Northern analysis at relapse compared with presentation. We note that the concentrations of RA that are achieved in human plasma (Fig 1 and Table 1; 300 μg/mL = 1 μmol/L) are quite low relative to concentrations that have been used for continuous cell cultures in vitro. Resolution of this issue will depend upon sequence analysis of PML/RAR-α in larger numbers of patients now in progress, but to date this explanation appears doubtful.

A third possibility is that enzymes responsible for catabolic drug conversion are induced by continuous treatment. A cytochrome P450-like enzyme system catalyzes the oxidation of RA that leads to the formation of 4-oxo-all-trans RA. Phenobarbital, a prototype drug that induces its own catabolism by induction of the P450 system, markedly increases the plasma clearance of both all-trans RA and 13-cis RA; yet repeated treatment with 13-cis RA does not induce the pharmacokinetic behavior we describe for all-trans RA. Phenobarbital-induced accelerated catabolism can be overcome by simply increasing the drug dose; however, a twofold dose increase of RA in six of our patients not only failed to recapture their clinical response, but also failed to significantly increase plasma drug concentrations.

Although the inability to present an effective drug concentration to leukemic cells in plasma may be a primary mechanism for relapse from RA-induced remissions, the low plasma concentrations do not necessarily imply pharmacologic inactivity in other tissues. One of our patients experienced marked drug-related toxicity (pseudotumor cerebri) during a period when her plasma concentrations were undetectable by HPLC (R. Warrell Jr, unpublished observations). Thus, accelerated enzymatic degradation may not constitute the complete pharmacologic explanation for this phenomenon. An important additional factor may be that RA increases the expression of CRABP in normal body tissues. Several studies have shown that repeated topical application of all-trans RA increases CRABP in human skin. Epidermal CRABP levels were also found to increase after systemic administration of acitretin but not after treatment with 13-cis RA. One possible consequence of a generalized increase in CRABP would be that normal tissues could act as a large “retinoid sink,” resulting in accelerated clearance and very low plasma concentrations after continuous therapy, as observed in this study. Increased CRABP within the leukemic cell could also act to sequester RA intracellularly and prevent its transport to the nucleus; however, other human myeloid leukemic cells (HL-60 and KG-1) selected for resistance to all-trans RA do not show an increase in CRABP expression (nor does it appear that CRABP mediates RA-induced differentiation of these cells). Moreover, leukemic cells obtained from four of our patients who were clinically resistant retained in vitro sensitivity to the morphologic differentiating activity of the drug. Similar to other studies, these experiments were conducted using a single, relatively high concentration of RA (10⁻⁶ mol/L). Retinoid “resistance” is not likely an all-or-none phenomenon and more detailed studies may show a shift in the concentration/response curve.

If induced accelerated catabolism is the principal mechanism that underlies the progressive reduction in plasma concentrations, coadministration of a P450 enzyme inhibitor could ameliorate this problem. If increased CRABP expression in normal cells is primarily responsible, intermittent treatment (eg, dosing on alternate weeks, alternate months, etc) might minimize the effect because downregulation of the protein may occur rapidly after removal of the stimulus. Alternatively, because this effect has not been reported for other retinoids, other compounds may need to be identified that share the exquisite therapeutic sensitivity for the disease but do not upregulate CRABP. Studies that evaluate the time-course of CRABP expression in vivo during chronic RA treatment, as well as the use of P450 inhibitors, are currently in progress. Strategies to overcome these pharmacologic effects are clearly desirable before initiating large-scale studies that use chronic dosing with all-trans RA in patients with APL and other cancers.

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