Constitutive Expression of Cellular Retinoic Acid Binding Protein II and Lack of Correlation with Sensitivity to All-Trans Retinoic Acid in Acute Promyelocytic Leukemia Cells

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ABSTRACT

The up-regulation of cellular retinoic acid binding protein-II (CRABP-II) has been invoked as an important mechanism of clinically acquired resistance to all-trans retinoic acid (RA) therapy in acute promyelocytic leukemia (APL). To test this hypothesis, we used quantitative reverse transcription-PCR and fast performance liquid chromatography procedures to examine the levels of CRABP-II mRNA and RA binding activity in APL patient samples. We found that CRABP-II mRNA in APL cells from pretreatment patients (n = 36) was constitutively expressed at relatively high levels (median, 0.92; range, 0.16-4.13) relative to the level in CRABP-II protein-expressing NB4 cells (arbitrarily set at 1.0 unit). Consistent with this finding, the RA binding activity of CRABP in APL cells from three pretreatment cases (range, 27.2-53.2 fmol/mg protein) was similar to that of NB4 cells (22.6 ± 5.4 fmol/mg protein). Furthermore, in the pretreatment samples, there was no association between CRABP-II mRNA expression level and APL cellular sensitivity to RA-induced differentiation in vitro. After 45 days of remission induction therapy on Eastern Cooperative Oncology Group protocol E2491, CRABP-II mRNA was modestly increased from day 0 values in patients treated with either RA (median increase, 0.41) or chemotherapy (median increase, 0.56), and there was no significant difference between the two treatment groups (P = 0.91). In patients studied after relapse from RA therapy (n = 7), there was a significant decline in APL cell sensitivity to RA-induced differentiation in vitro compared with patients after relapse from chemotherapy (n = 5; P = 0.015-0.055 at three RA concentrations tested), but in the RA relapse cases, there was no change from pretreatment levels of CRABP-II mRNA (median, 0.98) or, in three relapse cases studied, of RA protein binding activity (range, 22.1-70.7 fmol/mg protein). Taken together, our data strongly imply that variations in CRABP-II expression and RA binding activity are not causally related to the development of clinically acquired APL cellular RA resistance, but rather, they suggest that constitutive expression of CRABP-II could have a facilitative role in the response of APL cells to RA.

INTRODUCTION

RA induces terminal differentiation of APL cells (1-3) and is highly effective in inducing complete hematological and CR in APL patients (4-8). However, the duration of the CR with RA as a single agent is usually brief, averaging about 3 months (4-7). Furthermore, patients who relapse from previous RA therapy, even after consolidation/maintenance chemotherapy, have usually shown a poor response to retreatment with RA, indicating the development of acquired clinical RA resistance (6, 7, 9).

The mechanism of acquired clinical RA resistance in APL patients has been related to alterations of RA pharmacokinetics (10, 11). In virtually all cases, the Cmax and the area under the concentration-time curve for plasma levels of RA are decreased by >50% within 1 week of beginning RA therapy (11, 12). This decrease is, at least partly, due to increased RA oxidation by cytochrome P-450 and lipoxygenase enzymes (12, 13). However, these systemic changes cannot explain frequent observations of reduced sensitivity of APL cells to RA-induced differentiation in vitro after relapse from RA therapy, as indicated by a right shift of the dose-response curve (6, 9, 14, 15). This suggests the involvement of an intrinsic leukemic cellular mechanism(s). This could be due to inactivating mutations in the RARe or PML-RARα genes as found in retinoid-responsive human myeloid leukemia cell lines HL60 (non-APL) and NB4 (APL) cells after selection for resistance to RA (16-19). Indeed, we recently found missense mutations in the RARα region of PML-RARα fusion gene in 3 of 11 APL patients who relapsed after nonconcurrent RA + chemotherapy on protocol E2491 (15). This suggests that intrinsic, genetic alterations in PML-RARα, the primary RA target gene in APL cells, likely accounts for some cases of clinical RA resistance, but it does not account for the APL cellular resistance observed in the majority of APL relapse cases. Another mechanism has been implicated by French investigators: increased expression of CRABP-II in APL cells (14, 20, 21). These investigators reported that RA binding activity of CRABP-II was not detectable in APL cells from pretreatment patients but that moderately increased levels were detectable in bone marrow aspirate cells after in vivo treatment with RA for 30-90 days, and most importantly, that considerably increased levels were detectable in four patients who relapsed from CRs achieved with RA-containing treatment regimens (14). After systemic RA treatment, CRABP RA binding levels were also reported to be increased in primate skin cells (11), and this likely is predominantly CRABP-II, because CRABP-II mRNA, but not CRABP-I mRNA, was markedly increased in human skin after topical RA application (22). These observations were consistent with the more general hypothesis that exposure to high levels of RA can activate a feedback protective mechanism in which increased RA levels activate nuclear RARs to interact with a RA response element in the promoter region of the CRABP-II gene (23, 24), leading to increased levels of this cytoplasmic protein. This induced CRABP-II protein is then hypothesized to sequester RA in the cytoplasm, specifically targeting it to catabolic oxidative enzymes associated with the endoplasmic reticulum and decreasing the delivery of free RA to nuclear RAR transcription factors. Such a negative feedback mechanism might contribute to clinical RA resistance both by inducing a systemic sump for RA sequestration (skin and other cell
types) and by intrinsically reducing APL cellular responsiveness to RA (11, 14, 20, 21).

The current study was initiated to determine whether interpatient variations in the putative inductive response of CRABP-II to RA in APL cells might be related to differences in clinical outcome on protocol E2491 in which previously untreated APL patients were randomized to remission induction therapy with either RA or chemotherapy (8). Surprisingly, however, we found that CRABP-II mRNA and CRABP RA binding activity were constitutively expressed in APL cells and that they manifested only relatively minor changes during remission induction therapy or at relapse. Our data suggest that, despite the plausibility of the CRABP-II negative feedback hypothesis, it does not appear to be an important factor in the development of APL cellular resistance to RA.

MATERIALS AND METHODS

Patient Samples. We investigated 125 leukemic cell samples obtained from 42 APL patients. Forty patients were treated under Eastern Cooperative Oncology Group protocol E2491. Two patients from the Department of Oncology, Montefiore Medical Center, were treated with RA according to the RA induction therapy arm of protocol E2491 (8). Diagnostic criteria for APL were those of the French-American-British classification (25), and all cases included in the present study were confirmed by the presence of the PML-RARA fusion gene (26). As detailed elsewhere (8), protocol E2491 involved a double randomization: remission induction with either RA (45 mg/m² daily for 45 days) or chemotherapy (45 mg/m² daunorubicin i.v. on days 1–3 plus 100 mg/m² cytarabine i.v. on days 1–7) and, following two rounds of consolidation chemotherapy (first as induction; second, 45 mg/m² daunorubicin i.v. on days 1–2 plus 2 g/m² cytarabine every 12 h × 8), maintenance with RA, 45 mg/m² daily for 1 year, or observation. Thirty six samples were collected on day 0 (pretreatment), 26 on day 15, 17 on day 30, 32 on day 45, and 14 at relapse. The median age of the patients was 37 years, with a range of 17–73 years. There were 18 males and 24 females. PML-RARA typing showed that 17 patients were S-form, 19 patients were L-form, and 6 patients V-form. The diagnosis of APL was confirmed by the presence of the PML-RARα fusion gene (22, 29). Due to the high sequence homology between the CRABP-II and CRABP-1 genes, we demonstrated the specificity of the reaction by endonuclease digestion of the PCR product amplified using these two primers. CRABP-II cDNA contains a PstI site between nucleotide 206 and 207, which is absent in the CRABP-I gene.

PCR amplifications were performed under approval of the Institutional Review Boards of all participating institutions. A low-density WBC fraction (density ≤1.077 g/ml) was prepared by standard sodium metrizamide step gradient centrifugation. Cells were harvested for cytospin and cytological analysis, RNA extraction, and short-term suspension culture with varying concentrations of RA.

Cell Lines. The human myeloid leukemia cell lines HL-60 (27) and NB4 (a gift from Dr. M. Lanotte, Paris, France; Ref. 28) were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO2.

RNA Extraction and Reverse Transcription. Total cellular RNA was prepared by the guanidine isothiocyanate extraction-cesium chloride gradient ultracentrifugation procedure or using a commercial kit according to the supplier’s direction (TRizol, Life Technologies, Gaithersburg, MD). Six µg of total cellular RNA were denatured at 75°C in 36 µl of diethyl pyrocarbonate treated H2O for 10 min and chilled on wet ice. RT was done in a 60-µl total volume with 150 pmol of random hexamers (pdN6; Pharmacia, Piscataway, NJ), 500 µM deoxynucleotide triphosphates (Perkin-Elmer, Norwalk, CT), 42 units of RNase inhibitor (RNAsin, Promega, Madison, WI), and 15 units of Superscript RT (Life Technologies, Inc.) in 1× PCR buffer (1.5 mM MgCl2, 50 mM KCl, and 10 mM Tris-HCl, pH 8.3). The reaction mixtures were kept at room temperature for 10 min, followed by incubation at 41°C for 1 h. RT reactions were terminated by heating to 95°C for 5 min. cDNA was stored in aliquots of 1 µg of RNA equivalent, each at −20°C.

Quantitative RT-PCR. cDNA equivalent to 0.2 µg of total RNA and an equicompetitive amount of CRABP-II mimic DNA (10⁻³ amol) were prepared as described above, were coamplified using primers 5’-CCTCTGCGAACTG-GAAAAATCA (sense strand) and 5’-CTCTCTCCACACCTTGAGG (antisense strand). They span a 201-bp fragment corresponding to nucleotides 110–311 of the CRABP-II cDNA sequence encoded by exons 1 and 2 of the CRABP-II gene (22, 29). Due to the high sequence homology between the CRABP-II and CRABP-I genes, we demonstrated the specificity of the reaction by endonuclease digestion of the PCR product amplified using these two primers. CRABP-II cDNA contains a PstI site between nucleotide 206 and 207, which is absent in the CRABP-I gene.

PCR amplifications were performed on 3% Nusieve agarose gels (FMC, Rockland, ME) and stained with ethidium bromide. Band intensities were determined from measurement of photographic negatives of ethidium bromide-stained gels, using densitometry and digitized computer analysis with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). All measurements of CRABP-II were normalized to G3PDH to correct intraassay variation. The CRABP-II mRNA level of each sample was expressed relative to that of NB4 cells in the following manner:

\[
\text{Sample CRABP-II cDNA/Mimic ratio} = \left( \frac{\text{NB4 CRABP-II cDNA/Mimic ratio}}{\text{Sample G3PDH cDNA/Mimic ratio}} \right)
\]

In pilot experiments, we determined that the ratio of the signal intensity of the Mimic DNA-templated to the endogenous cDNA-templated PCR products provide an accurate estimate of the relative amounts of the two templates at the equivalence point ±1 log (data not shown; Ref. 30).

Construction of Competing Templates (Mimics). CRABP-II and G3PDH Mimics were generated using the Mimic protocol from Clontech (Palo Alto, CA; Ref. 31). Briefly, using a 633-bp BamHI/EcoRI fragment of v-erb B2 gene as template, a PCR was performed using a pair of oligonucleotide primers that consist of a 20-mer CRABP-II gene-specific oligonucleotide (underlined) linked at its 3'-end with a 20-mer v-erb B2-specific oligonucleotide (sense, 5’-CCTGCGAACTG-GAAAAATCCAAGTTGCTGAGC-3’; antisense, 5’-CCTCTCCACACCTTGAGG (10⁻³ amol) and 15 units of Superscript RT (Life Technologies, Inc.) in 1× PCR buffer (1.5 mM MgCl2, 50 mM KCl, and 10 mM Tris-HCl, pH 8.3). The reaction mixtures were kept at room temperature for 10 min, followed by incubation at 41°C for 1 h. RT reactions were terminated by heating to 95°C for 5 min. cDNA was stored in aliquots of 1 µg of RNA equivalent, each at −20°C.

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\]
coefficient was used to evaluate the correlation between two outcomes (33).

Concentrations of RA (10 nM, 100 nM, and 1 uM), which was dissolved in cytosolic extracts prepared from NB4 cells showed the presence of a Myeloid Leukemia Cell Lines. Using quantitative RT-PCR over a 3 values were evaluated by the sign rank test (33). The Spearman correlation summarize data. Differences between two treatment groups were evaluated by not normally distributed. The median and range of values were used to be determined by the NET dye reduction test (Sigma Chemical Co., St. Louis, MO).

**RESULTS**

Expression of CRABP-II mRNA and RA Binding Activity in Myeloid Leukemia Cell Lines. Using quantitative RT-PCR over a 3 log range of added CRABP-II DNA Mimic, we demonstrated that NB4 cells express CRABP-II mRNA at an ~8-fold higher level than HL-60 cells (Fig. 1). As measured by FPLC size-exclusion analysis, cytosolic extracts prepared from NB4 cells showed the presence of a specific binding peak at fraction number 32, corresponding to a molecular mass of slightly less than 18 kDa (Fig. 2A), which is consistent with the size of CRABP as reported for FPLC/HPLC analysis after [3H]RA binding (32, 34). The RA binding activity of this peak was 22.60 ± 5.44 fmol/mg protein (see also Table 3). No specific [3H]RA binding activity was detected in the cytosolic extracts from HL-60 cells (Fig. 2B). Therefore, NB4 cells were used as a positive control for measuring CRABP-II expression in APL patient samples.

**CRABP-II mRNA Expression in Pretreatment and Postinduction Therapy Patient Samples.** CRABP-II mRNA determinations from two APL patients prior to and at various time points of RA therapy or chemotherapy are representatively illustrated in Fig. 3, and the overall results are summarized in Table 1. In pretreatment RA-naive cases (n = 36), CRABP-II mRNA was constitutively expressed at an indistinguishable median level (median, 0.92; range, 0.16–4.13) from that in CRABP-II protein-expressing NB4 cells (arbitrarily set at 1.0). After beginning RA therapy (26 patients), the median CRABP-II mRNA level at day 15 was 1.03 and at days 30 and 45 days were 1.19 and 1.42, respectively. For the chemotherapy group (16 patients), these values were 1.11, 0.86, and 1.22 at days 15, 30, and 45, respectively. In the subset of RA-treated (n = 18) and chemotherapy-treated (n = 9) patients in which matched pretreatment and day 45 posttreatment samples were available, there was no statistical difference in the change in CRABP-II mRNA levels between these two treatment groups (Table 2; P = 0.91; Wilcoxon rank-sum test). Using the combined results from the RA-treated and chemotherapy-treated
CRABP-II mRNA Expression

Fig. 3. Representative illustration of competitive RT-PCRs for quantitative measurement of CRABP-II mRNA in APL patient samples obtained at different time points of treatment. A, CRABP-II mRNA expression relative to CRABP-II Mimic. B, parallel RT-PCR for G3PDH mRNA expression to normalize CRABP-II results. Lane M, molecular weight standards; Lane O, blank control (no cDNA or Mimic). PKx, pretreatment; Rei, relapse; P, peripheral blood; B, bone marrow.

Table 1 Relative CRABP-II mRNA expression in APL samples before treatment, after induction therapy with RA or chemotherapy, and at relapse

<table>
<thead>
<tr>
<th>Pretreatment samples</th>
<th>n</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>36</td>
<td>0.92</td>
<td>0.16-4.13</td>
</tr>
<tr>
<td>RA therapy samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>23</td>
<td>0.89</td>
<td>0.16-2.25</td>
</tr>
<tr>
<td>Day 15</td>
<td>20</td>
<td>1.03</td>
<td>0.35-3.53</td>
</tr>
<tr>
<td>Day 30</td>
<td>13</td>
<td>1.19</td>
<td>0.43-3.27</td>
</tr>
<tr>
<td>Day 45</td>
<td>21</td>
<td>1.42</td>
<td>0.64-5.43</td>
</tr>
<tr>
<td>Relapse*</td>
<td>7</td>
<td>0.98</td>
<td>0.51-3.21</td>
</tr>
<tr>
<td>Chemotherapy samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>13</td>
<td>0.95</td>
<td>0.27-4.13</td>
</tr>
<tr>
<td>Day 15</td>
<td>6</td>
<td>1.11</td>
<td>0.57-2.71</td>
</tr>
<tr>
<td>Day 30</td>
<td>4</td>
<td>0.86</td>
<td>0.29-1.48</td>
</tr>
<tr>
<td>Day 45</td>
<td>11</td>
<td>1.22</td>
<td>0.79-3.46</td>
</tr>
<tr>
<td>Relapse*</td>
<td>6</td>
<td>0.89</td>
<td>0.47-3.57</td>
</tr>
</tbody>
</table>

* Treated with RA during either the induction or maintenance phases of protocol E2491.

Correlative Analysis of CRABP-II mRNA Levels and Sensitivity to RA-induced Differentiation in Pretreatment APL Cells. We investigated whether there was any association in pretreatment APL cells between the level of CRABP-II mRNA and APL cellular sensitivity to RA-induced differentiation in vitro. As shown in Fig. 5, there were no significant correlations between the CRABP-II mRNA level and two different measures of APL cell differentiation, the percentage of NBT-positive or of CD11b-positive cells after exposure to an inducing concentration of RA (100 nM) for 5 days in vitro.

CRABP-II mRNA and RA Binding Activity in APL Cells after Relapse from RA Therapy. By our quantitative RT-PCR assay, the median relative CRABP-II mRNA level in seven patients who relapsed after receiving RA therapy during either the induction and/or

Table 2 Changes of CRABP-II mRNA expression in APL samples at day 45 of treatment or at relapse compared with matched day 0 samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>δ median</th>
<th>δ range</th>
</tr>
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<tbody>
<tr>
<td>RA day 45 to day 0</td>
<td>18</td>
<td>0.41</td>
<td>-0.16-4.23</td>
</tr>
<tr>
<td>Chemotherapy day 45 to day 0</td>
<td>9</td>
<td>0.56</td>
<td>-0.11-2.67</td>
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<tr>
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<td>0.19</td>
<td>-0.36-2.17</td>
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<tr>
<td>Chemotherapy Relapse to day 0</td>
<td>5</td>
<td>-0.54</td>
<td>-3.04-3.3</td>
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* Comparison by Wilcoxon rank-sum test: P = 0.91 for δ1 versus δ2; P = 0.272 for δ3 versus δ4.

patients in which matched pretreatment and day 45 samples were available (n = 27), there was a significant increase in the CRABP-II mRNA level after 45 days of either therapy (P = 0.001; sign rank test).

CRABP RA Binding Activity in Pretreatment Patient APL Cells. To exclude the possibility that CRABP-II mRNA may not be translated to functional CRABP protein, the [3H]RA binding activity of cytotoxic extracts of these cells was measured by FPLC size-exclusion analysis. A specific binding peak at fraction number 32 corresponding to a molecular mass of ~18 kDa was found in the FPLC profile (Fig. 4A), which is similar to that for NB4 cells (Fig. 2A). Furthermore, the amount of RA bound by the protein peak in pretreatment APL cells was similar to that bound by NB4 cells (Table 3). Somewhat lower values were recorded for the fresh APL cell RA binding activity on a cell count basis (5.0-8.5 fmol versus 13.6 fmol per 10⁶ cells), probably related to the larger size of NB4 cells, whereas slightly higher values were recorded based on protein amount (27.2-53.2 fmol versus 22.6 fmol/mg protein).

Table 3 CRABP RA Binding Activity in Pretreatment APL Cells

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DISCUSSION

The hypothesis that up-regulation of CRABP-II by pharmacological concentrations of RA causes clinically relevant APL cellular RA resistance implies that the cellular level of CRABP-II expression should be directly related to RA exposure and inversely related to APL cell sensitivity to RA-induced differentiation. The present study provided essentially four pieces of evidence contrary to these expectations: (a) CRABP-II mRNA and CRABP RA binding activity were constitutively expressed in APL cells from all tested cases of previously untreated, RA-naive patients; (b) we found no correlation between the expression level of CRABP-II mRNA and the sensitivity of APL cells to RA-induced differentiation in vitro; (c) we found a <2-fold increase in CRABP-II mRNA expression level in APL cells from patients during the remission induction phase of treatment on protocol E2491 that did not differ between RA- and chemotherapy-treated patients, suggesting that the modest change might be related to a shift in cell population after treatment; (d) despite a loss of sensitivity to RA-induced differentiation of APL cells from patients who relapsed after RA therapy, there was no significant difference in these cells of the expression level of CRABP-II mRNA. From analysis of three pretreatment and three relapse cases, there was also no indication of any difference in the level of CRABP RA binding activity between these time points. Furthermore, we found similar levels of maintenance phases of protocol E2491 was 0.98 versus 0.89 for six relapse patients who had received only chemotherapy (Table 1). Statistical evaluation of the six RA-treated and five chemotherapy-treated patients for whom matched pretreatment and relapse samples were available indicated no significant change in the CRABP-II mRNA level after treatment ($P = 0.744$) and no difference between the two treatment groups ($P = 0.272$; Table 2). Furthermore, the cytosolic RA binding activity in relapse APL cells (Fig. 3B) appeared virtually identical to that from pretreatment APL cells (Fig. 3A) and NB4 cells (Fig. 2A), and the range of $[^{3}H]RA$ binding activity in the relapse cells was overlapping with that from pretreatment cells (Table 3).

Loss of APL Cell Sensitivity to RA-induced Differentiation in Vitro after Relapse from RA Therapy. Tests for sensitivity to RA-stimulated differentiation in vitro were performed on matched pretreatment and relapse samples from seven RA-treated and five chemotherapy-treated protocol cases analyzed for CRABP-II mRNA levels. As summarized in Fig. 6, there was a significant reduction in the percentage of NBT-positive cells from pretreatment values at all three RA concentrations tested in patients who relapsed after RA therapy compared with patients who relapsed after chemotherapy alone.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak CRABP/10^7 cells</th>
<th>Peak CRABP/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB4</td>
<td>1107 dpm (14.1 fmol)</td>
<td>1767 dpm (22.5 fmol)</td>
</tr>
<tr>
<td>NB4</td>
<td>1116 dpm (14.2 fmol)</td>
<td>2214 dpm (28.1 fmol)</td>
</tr>
<tr>
<td>Mean</td>
<td>3978 (12.4 fmol)</td>
<td>1358 dpm (17.3 fmol)</td>
</tr>
<tr>
<td>± SD</td>
<td>1067 ± 77 dpm (13.6 ± 1.0 fmol)</td>
<td>1780 ± 428 dpm (22.6 ± 5.4 fmol)</td>
</tr>
<tr>
<td>APL pretreatment</td>
<td>430 dpm (5.5 fmol)</td>
<td>4056 dpm (51.5 fmol)</td>
</tr>
<tr>
<td>APL pretreatment</td>
<td>667 dpm (8.5 fmol)</td>
<td>4191 dpm (53.2 fmol)</td>
</tr>
<tr>
<td>APL pretreatment</td>
<td>396 dpm (5.0 fmol)</td>
<td>2141 dpm (27.2 fmol)</td>
</tr>
<tr>
<td>APL relapse</td>
<td>229 dpm (2.9 fmol)</td>
<td>3907 dpm (49.6 fmol)</td>
</tr>
<tr>
<td>APL relapse</td>
<td>185 dpm (2.4 fmol)</td>
<td>5570 dpm (70.7 fmol)</td>
</tr>
<tr>
<td>APL relapse</td>
<td>344 dpm (4.4 fmol)</td>
<td>1741 dpm (22.1 fmol)</td>
</tr>
</tbody>
</table>

CRABP-II mRNA expression in two normal bone marrow specimens to that in NB4 cells,\(^4\) such that the rather wide range of CRABP-II mRNA levels noted in APL patient samples before and after therapy (0.16–5.43 relative to 1.0 for NB4 cells) cannot be attributed to variable admixture with normal cellular elements. That these values reflect true variations in intracellular expression levels is supported by the considerations that all CRABP-II mRNA determinations were normalized to known quantities of competitor DNA for both CRABP-II and G3PDH and that independently transcribed and amplified triplicate NB4 RNA controls in each experiment showed <10% variation from the mean.

We have no satisfactory explanation for the differences between our findings and those of previous reports, particularly the discrepancy between our finding of constitutive versus absent (14, 20) CRABP expression in pretreatment, RA-naive APL cells (or in normal bone marrow cells). At the protein level, perhaps it is partly related to technical differences, because earlier reports used a gel electro-

\(^4\) Our unpublished results.
CRABP-II is neither sufficient nor essential for leukemic cellular CRABP-II expression in RA-sensitive HL-60 cells (41), in agreement with the induction or maintenance phases of protocol E2491 (8). Horizontal lines, median values. The Wilcoxon rank-sum test was used to evaluate Ps.

More generally, the notion that a CRABP-II-mediated negative feedback mechanism has an important role in APL cellular RA resistance was founded on the assumption that CRABP-II and CRABP-I have similar functions, although the crucial preexisting experimental data relating CRABP expression level to RA cytoplasmic sequestration and enhanced oxidative metabolism was developed only with CRABP-I (44, 45). This notion seemed plausible because the unique RA response element in the CRABP-II promoter region (23, 24) might reasonably activate a negative feedback response in the presence of increased RA and because the CRABP-I and -II are quite similar in both their primary and tertiary structures. Each is composed of 136 amino acids and displays a flattened globular domain consisting of two orthogonal β-sheets that provide a sandwich-like binding pocket for RA with its cyclohexenyl ring oriented to the protein interior (reviewed in Ref. 46). However, a number of observations suggest that CRABP-I and CRABP-II may have important functional differences. Although each is highly conserved during evolution, the two proteins are only 73% homologous, with most differences related to hydrophilic residues on the protein surface that likely differentially affect interactions with other intracellular molecules (22, 46). CRABP-II has been variously assessed to bind RA 3- to 15-fold less avidly than CRABP-I (47, 48). Although both CRABP-I and -II transcripts and proteins are widely expressed during both pre- and postnatal life in a time-dependent manner, they are most frequently expressed with nonoverlapping tissue and/or cell specificities (35, 39, 46, 49). Moreover, there is evidence that the regulation and possibly the function of CRABP-I and CRABP-II may vary in different cell types (22, 39, 46). Thus, although our results seem to exclude a negative feedback mechanism for CRABP-II in APL cells, this does not rule out such a role in other cell types that might contribute to the systemic alterations in RA metabolism in RA-treated APL patients (10, 11, 21).

ACKNOWLEDGMENTS

We thank Dr. David Ong (Vanderbilt University, Nashville, TN) for allowing us to mention his unpublished results and for critical reading of the manuscript. Dr. Elisabeth Paietta (Albert Einstein Cancer Center, Bronx, NY) for the provision of CD11b data, Dr. Clara Nervi (University of Rome, Rome, Italy) for advice regarding the performance of [3H]RA binding assays, and Dr. Paul Siebert (Clontech, Palo Alto, CA) for the gift of v-erb B2 DNA. We also thank the physicians, nursing staffs, and data managers of Eastern Cooperative Oncology Group affiliated institutions for their cooperation in obtaining the clinical specimens used for these studies.

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Da-Cheng Zhou, Steven J. Hallam, Sandra J. Lee, et al.

*Cancer Res* 1998;58:5770-5776.

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