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 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 lipoxigenase 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 RARα 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 median age of the patients was 37 years, with a range of 17–73 years.

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 pyrocarboxylic treated H₂O for 10 min and chilled on wet ice. RT was done in a 60-/xl 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 (RNAin, 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 at −20°C.

Quantitative RT-PCR. cDNA equivalent to 0.2 µg of total RNA and an equimultiplicative amount of 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 PoI site between nucleotide 206 and 207, which is absent in the CRABP-I gene.

PCRs in 50 µl contained 0.2 µg of RNA-equivalent cDNA, 10 pmol of each primer, 150 µM of each deoxynucleotide triphosphate, 5 µl of 10× PCR buffer [Perkin-Elmer; 500 mM KCl, 100 mM Tris-HCl (pH 8.5), 15 mM MgCl₂, and 0.1% gelatin] and 1.5 unit AmpliTaq Gold (Perkin-Elmer). The competitor template (Mimic) was added to the PCR master mix prior to aliquoting. Thirty-eight amplification cycles were performed at 94°C, 1 min; 57°C, 1 min; and 72°C, 1 min 30 s, followed by an additional 10 min at 72°C. Each PCR included negative controls containing water or Mimic alone and triplicate NB4 cDNA positive controls (each transcribed from an independent RNA aliquot) as an index of reaction tube variability.

For testing the integrity of the RNA and normalizing the intrasample variation of cDNA synthesis, competitive PCR was performed with the housekeeping gene G3PDH, using the following primers: sense, 5'-CATCTCTGGCCCCCCTTGCTG-3' at nucleotide 416; antisense, 5'-CCCTCCCGACGCCTGCTTCAC-3' at position 840. PCRs were performed with 0.1 µg of RNA-equivalent of cDNA and an equimultiplicative amount of G3PDH mimic (0.15 amol). PCR conditions were similar to that for amplification of CRABP-II, except for the annealing temperature (58°C) and cycle number (24 cycles).

PCR products were separated 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:

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

In pilot experiments, we determined that the ratio of the signal intensity of the Mimic DNA-templated to the endogeneous 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 (sense, 5'-CTTGGCAACTGGAATCCGACGCAAGTTTCTGAGC-3'; antisense, 5'-CTCCTCCCCCAACACCTGAAGTGGCCTCTTGCAATT-TCTGT-3'). The 350-bp PCR product thus obtained consists of a v-erb B2 DNA with 20-mer CRABP-II cDNA sequences at both ends. After purification using a spin-column (QIAquick Nucleotide Removal kit, Qiagen, Valencia, CA) and determination of the DNA concentration by UV spectrophotometry, the mimic was diluted, aliquoted, and stored at −20°C until use. The mimic for G3PDH was generated in the same manner, with the sense primer 5'-CATCTCTGGCCCCCCTTGCTGCTCCCCGATCTTGTTAT-3' and the antisense primer 5'-CCCTCCCGACGCCTGCTTCACA CCCTGCCATT CTGGT-3'.

Cytosolic Extract Preparation. Cytosolic extracts were prepared from NB4 and HL60 cells and from fresh blast cells of APL patients by the method of Jette et al. (52). Briefly, 2–5 × 10⁶ cells were washed twice with ice-cold PBS and once with PTG buffer (5 mM sodium phosphate (pH 7.4), 10 mM monothioglycerol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µM/ml aprotinin, and 10 µM/ml leupeptin). The cells were resuspended in 3–5 ml of PTG buffer and incubated on ice for 30 min, followed by homogenization in a glass Dounce homogenizer (pestle B). The homogenate was centrifuged for 15 min at 1000 × g. The supernatant is transferred to a fresh tube and centrifuged again for 30 min at 130,000 × g using a 50Ti rotor (Beckman, Palo Alto, CA).

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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

[Image of Graph: Ratio of CRABP-II mRNA/Mimic vs. CRABP-II Mimic added (amol)]

Coefﬁcient was used to evaluate the correlation between two outcomes (33). Concentrations of RA (10 nM, 100 nM, and 1 µM), which was dissolved in cytosolic extracts prepared from NB4 cells showed the presence of a

Wilcoxon rank-sum test (33). Changes in pretreatment to posttreatment were not normally distributed. The median and range of values were used to summarize data. Differences between two treatment groups were evaluated by

Statistical Methods. Nonparametric methods were used, because the data were not normally distributed. The median and range of values were used to summarize data. Differences between two treatment groups were evaluated by the Wilcoxon rank-sum test (33). Changes in pretreatment to posttreatment values were evaluated by the sign rank test (33). The Spearman correlation coefficient was used to evaluate the correlation between two outcomes (33).

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.

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Fig. 1. CRABP-II mRNA expression in NB4 and HL60 cell lines determined by competitive RT-PCR. cDNA equivalent to 0.2 µg of total RNA was amplified in the presence of an increasing amount of CRABP-II Mimic ranging from 1 log lower to 1 log higher than the equicompetitive point (0.0 ratio on ordinate). PCR products were separated on ethidium bromide-stained gels, and the ratios of band intensities of endogenous CRABP-II compared with CRABP-II Mimic were measured from photographic negatives using densitometry and digitized computer analysis.

Fig. 2. Size-exclusion FPLC analysis of cytosolic extracts prepared from NB4 cells (A) and HL60 cells (B). Extracts (250 µl) were incubated with 10 nM [3H]RA in the absence (O) or presence (%) of 200-fold excess of unlabeled RA for 18 h at 4°C. The samples were fractionated over a Superose 12 HR 10/30 size-exclusion column at 0.5-ml intervals. Arrows, the fraction numbers of marker proteins (in kDa) used to calibrate the FPLC column.
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

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 cytosolic 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 10^7 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).

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 0, blank control (no cDNA or Mimic). PKx, pretreatment; Rei, relapse; P, peripheral blood; B, bone marrow.

Table 1 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>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>0.35–3.55</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>
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</table>

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

†Not treated with RA during either the induction or maintenance phases of protocol E2491.
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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 CRABP-II mRNA expression in two normal bone marrow specimens to that in NB4 cells,

specific cytosolic RA binding activity was determined after incubation with 10 nM \(^{3}\text{H}\)RA ± a 200-fold excess of cold RA, followed by FPLC size-exclusion chromatography, as described in "Materials and Methods."

Table 3 CRABP-II RA binding activity in NB4 cells, pretreatment APL cells, and relapse APL cells from RA-treated patients

Specific cytosolic RA binding activity was determined after incubation with 10 nM \(^{3}\text{H}\)RA ± a 200-fold excess of cold RA, followed by FPLC size-exclusion chromatography, as described in "Materials and Methods."

Table 3 CRABP-II RA binding activity in NB4 cells, pretreatment APL cells, and relapse APL cells from RA-treated patients

<table>
<thead>
<tr>
<th>Sample/Case</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>NB4</td>
<td>978 dpm (12.4 fmol)</td>
<td>1385 dpm (17.3 fmol)</td>
</tr>
<tr>
<td>Mean ± 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>

Fig. 5. Correlative analysis of the relative expression level of CRABP-II mRNA with the percentage of NBT-positive (A) or CD11b-positive (B) cells in pretreatment APL samples. The percentages indicate net NBT- and CD11b-positive cells determined by subtracting the percentage of positive cells cultured in the absence of RA from those cultured in the presence of 100 nM RA. r, Spearman correlation coefficient.

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-naïve 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 responses (32, 42), indicating that the expression of CRABP-II in RA-sensitive HL-60 cells (41), in agreement with previous reports (36-39), i.e., there is no precedent for discordant transcriptional/translational regulation of this protein. Furthermore, in NB4 cells, the cytoplasmic RA binding protein activity was demonstrated to react specifically with antibodies to CRABP-II. This cytoplasmic protein binding activity eluted from FPLC with a molecular mass of slightly less than 18 kDa, the appropriate size for CRABP. 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). Moreover, there is evidence that the regulation and possibly the function of CRABP-I and CRABP-II may vary in different cell types (22, 46). Therefore, 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 avidly than CRABP-I (47, 48). Although both CRABP-I and -II are expressed with nonoverlapping tissue and/or cell specificities (35, 39, 46, 49, 50). 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.

Our finding of constitutive CRABP-II expression in APL cells seems more consistent with the possibility that CRABP-II has a positive role in the cellular response to RA, as was recently demonstrated to be the case in several breast cancer cell lines (40). This also seems more consistent with the expression of relatively high levels of CRABP-II mRNA and CRABP RA binding activity in the APL cell line NB4 (Figs. 1 and 2A), which was originally isolated from a RA-refractory patient but which is sensitive to RA-induced differentiation (28). However, our findings of approximately equal levels of CRABP-II expression in relapse APL cells with reduced in vitro sensitivity to RA-induced differentiation and of very low/absent CRABP-II expression in RA-sensitive HL-60 cells (41), in agreement with previous reports (32, 42), indicate that the expression of CRABP-II is neither sufficient nor essential for leukemic cellular sensitivity to RA-induced differentiation. These observations seem consistent with conclusions from studies with cultured breast cancer cells that CRABP-II is facilitory rather than obligatory for RA responsiveness (40) and with the demonstration that normal development and protection from excessive RA occurs in CRABP-II and CRABP-I + II knock-out mice (43). Perhaps the rather wide individual variations in CRABP-II expression in the leukemic/hematopoietic cells of APL patients are related to such a nonobligatory role. Additional studies are needed to more precisely assess the intracellular distribution and potential functional capacity of CRABP-II protein expression in specific cell types in heterogeneous tissues, which may now become practically accomplishable with the availability of specific antisera.

Fig. 6. Changes of in vitro sensitivity to RA-induced differentiation in APL samples at relapse compared with matched pretreatment samples as determined by the NBT test. The horizontal lines, median values. The Wilcoxon rank-sum test was used to evaluate Ps.

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