Increased Expression of the Multidrug Resistance-Associated Protein Gene in Relapsed Acute Leukemia

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Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine relative levels of transcripts for MDR1 and the recently described multidrug resistance-associated protein (MRP) in normal lymphohematopoietic cells and in 62 bone marrow aspirates of newly diagnosed and recurrent acute leukemia. Levels of MRP expression in newly diagnosed AML samples were similar to those observed in normal bone marrow cells (CD34-negative and CD34-positive) and in unselected HL60 human promyelocytic leukemia cells, which were used as an internal control throughout this study. In contrast, samples of AML obtained at the time of relapse contained approximately twofold higher levels of MRP RNA (P < .01). Analysis of paired samples, the first obtained at diagnosis and the second at relapse, from 13 acute myelogenous leukemia (AML) and four acute lymphocytic leukemia (ALL) patients showed that MRP expression was increased at the time of relapse in greater than 80% of patients. In contrast, no consistent changes of MDR1 expression at relapse were observed. These results raise the possibility that increased MRP expression might contribute to leukemia relapse. This is a US government work. There are no restrictions on its use.

With current induction regimens, 60% to 80% of patients with newly diagnosed acute myelogenous leukemia (AML) or acute lymphocytic leukemia (ALL) achieve a complete remission (CR). Despite this initial success, only 20% to 30% of adult patients with AML and 10% to 20% of adult patients with ALL are cured.1-3 The remainder of patients either never achieve a CR or relapse after initially achieving a remission. These refractory or relapsed leukemias often respond poorly to chemotherapy and display resistance to a broad spectrum of antineoplastic agents. Identifying the molecular mechanisms responsible for the failure of chemotherapy and the emergence of multiple-drug resistance (MDR) in relapsed patients is an area of intensive investigation.

Several mechanisms have been proposed for the development of MDR. In vitro and in vivo, the best studied mechanism involves overexpression of the MDR1 gene product, P-glycoprotein (Pgp).4-6 While it has been relatively straightforward to prove that elevated expression of Pgp causes resistance to anthracyclines, anthracenediones, epipodophyllotoxins, vinca alkaloids, and other agents in drug-selected tissue culture cell lines, directly proving that Pgp expression plays a role in clinical drug resistance has been more difficult. For example, several studies have suggested that MDR1 expression is a prognostic factor in AML7-11 and ALL,12 whereas other studies have suggested that Pgp is rarely expressed in AML13,14 or ALL15 samples, even at the time of relapse. Furthermore, studies using modulators of Pgp have suggested that the effect of Pgp on anthracycline accumulation is modest even in those samples that contain significant levels of Pgp.16-19,21 Thus, the role of Pgp expression as a prognostic factor and as a cause for relapse in acute leukemia remains an area for active investigation.19-22

It was recently suggested that overexpression of another gene product, the multidrug resistance-associated protein (MRP), also contributes to MDR in tissue culture cells.23 The full-length cDNA for MRP was isolated from the multidrug resistant human small-cell lung cancer cell line H69AR. It appears to encode an adenosine triphosphate (ATP)-binding (drug)-transport protein that is distinct from Pgp.24 Overexpression of this gene has also been detected in several other MDR cell lines, including two independently derived Pgp-negative, doxorubicin-resistant HL60 human acute promyelocytic leukemia cell line variants (HL60/AR and HL60/Adr).25-28 In many of these MRP-overexpressing cell lines, drug accumulation was reduced, and/or intracellular drug distribution was altered. Furthermore, recent studies demonstrated that transfection and expression of a cDNA for MRP in cells increased the resistance of the transfected cells to natural product drugs, including doxorubicin, etoposide, and vincristine.29-31 Thus, overexpression of MRP can lead to an MDR phenotype in vitro. However, to date there has been no evidence for a role for MRP expression in clinical drug resistance.

In a recent study16 we used a functional assay to probe for Pgp function in bone marrow aspirates from adults with newly diagnosed and relapsed AML. Evidence of Pgp function (defined as a > 20% increase in nuclear daunorubicin accumulation in the presence of the Pgp modulator quinidine) could be detected in only 10% of cases of newly diagnosed AML, and the incidence of the Pgp phenotype did not appreciably increase at the time of relapse. These studies have now been extended by measuring expression of MDR1 and MRP mRNA by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in paired samples obtained from the same patients at the time of initial diagnosis and again after relapse. The observation that MRP expression increased in more than 80% of samples at the time of relapse compared with diagnosis raises the possibility that MRP might play a role in drug resistance in human acute leukemia.
MATERIALS AND METHODS

Cell lines and clinical samples. Wild-type HL60 cells derived from an AML patient were obtained from American Type Culture Collection (ATCC; Rockville, MD). HL60/AR cells, a Pgp-negative multidrug-resistant subline selected for Adriamycin resistance, were a gift from S. Grant (Columbia University, New York, NY). SW620 colon cancer cells were provided by S. Bates [National Cancer Institute (NCI), Bethesda, MD].

Bone marrow aspirates were obtained before chemotherapy at the time of initial diagnosis or at relapse. Immediately after harvest, marrow mononuclear cells were isolated by sedimentation on Ficoll-Hypaque gradients and solubilized in guanidinium thiocyanate under reducing conditions. The samples analyzed in the present study were selected from a larger group of samples based on their high percentages of blasts at diagnosis and relapse. Examination of cytospin preparations indicated that samples obtained at diagnosis contained an average of 85% blasts.

Normal bone marrows were obtained from the iliac crest of normal donors after informed consent under a human use protocol approved by the NCI. Bone marrow aspirates (8-10 mL) were collected in each of two 20-mL syringes containing preservative-free heparin (Lymphomed, Deerfield, IL) as an anticoagulant and then diluted in Hank's balanced salt solution (HBSS) without CaCl₂ or MgCl₂ (GIBCO, Grand Island, NY) containing penicillin and streptomycin. Cells with a density of 1.077 g/mL were collected and washed three times with HBSS after separation on a Ficoll-sodium diatrizoate gradient (LSM; Organon Teknika Corp, Durham, NC). The low density cells were diluted to 2 × 10⁶ cells per milliliter in an enriched Iscove's Modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (HyClone, Logan, UT) and were incubated in tissue culture flasks overnight in a humidified 37°C incubator with 5% CO₂. The nonadherent cells were collected and washed twice with HBSS. CD34⁺ cells were isolated by positive immunomagnetic selection using a high-gradient magnetic separation column and MiniMacs CD34 progenitor cell isolation kit (Miltenyi Biotec Inc, Sunnyvale, CA). The purity of the recovered cells was determined by staining with CD34 (anti-HPCA-2) fluorescein isothiocyanate (FITC; Becton Dickinson, San Jose, CA), which binds to a distinct epitope on the CD34 molecule. In the present studies, flow cytometry analysis showed that the purity of CD34⁺ cells in the CD34⁻ fraction was 90% to 98% and less than 1% in the CD34⁺ fraction.

Normal peripheral blood cells obtained from a healthy donor by Ficoll gradient separation were separated into lymphocyte subfractions as previously described.

Adult patients with AML (median age, 43 years; range, 18 to 75 years) were subsequently treated on protocol JH8410 (cytisine arabinoside [Ara-C]/daunorubicin/4'-[9-acridinylamino]methanesulfonanilide [MAMS]); or JH8914 [granulocyte-macrophage colony-stimulating factor (GM-CSF)/Ara-C/daunorubicin/etoposide] as recently described. Adult patients with ALL (median age, 30 years; range, 18 to 74 years) were treated with prednisone, vincristine, etoposide, and L-asparaginase followed by Ara-C and daunorubicin on protocol JH8802 as recently described. Patients with fewer than 5% blasts in the marrow and recovery of normal marrow function that persisted at least until the time of consolidation therapy were considered to have achieved CRs. Patients who died of toxic complications (infection or bleeding) before the time of expected marrow recovery were considered not eligible for evaluation. All other patients were considered nonresponsive (NR).

Quantitative RT-PCR. Total RNA was prepared according to the method of Chomczynski and Sacchi. RT-PCR was performed as previously described. Total RNA (1 µg) from each AML or ALL sample or each cell line was reverse transcribed in 20 µL of RT buffer [10 mM TRIS-HCl, pH 8.3; 50 mM KCl; 5 mM L-MgCl₂; 1 mM each of deoxyadenosine triphosphate (dATP), deoxyguanylic triphosphate (dTTP), deoxythymidine triphosphate (dTTP) and (deoxythymidine triphosphate (dTTP) containing 2 U/µL RNase inhibitor, 0.003 A₅₅₀ units random hexanucleotides (both from Boehringer Mannheim, Indianapolis, IN), and 0.4 U/µL AMV reverse transcriptase (Promega, Madison, WI). The reaction conditions were 25°C for 10 minutes, 42°C for 15 minutes, and 95°C for 5 minutes. The resulting cDNA mixture was diluted twofold, fivefold, and 50-fold (or twofold, 10-fold, and 100-fold) in RT buffer. Target sequences for MDR1, MRP, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were separately amplified in the twofold, fivefold, and 50-fold dilutions, respectively, for 25 cycles (95°C for 10 seconds and 60°C for 15 seconds in a 9600 Thermocycler [Perkin-Elmer Cetus, Norwalk, CT]) by the PCR with specific primers for each gene, using the hot start modification. Digoxigenin-11-β-d-galactopyranoside-5′-triphosphate (0.1 µL; Boehringer Mannheim) was added to each tube of PCR mixture containing 12.5 mM/L TRIS, pH 8.3; 62.5 mM/L KCl; 2.5 mM/L MgCl₂; 0.2 mM/L each of dATP, dGTP, dCTP, and dTTP; 2.5 U/µL Taq polymerase (Boehringer Mannheim); and 0.3 mM/L of each specific primer pair in a total volume of 100 µL. An aliquot (15 µL) of the PCR products were separated on a 2% agarose gel in TRIS-borate-EDTA buffer. The gels were denatured and neutralized, and the DNA was transferred in 10 × SSC (1.5 mol/L sodium chloride, 150 mM/L sodium citrate solution) onto a nylon membrane (Boehringer Mannheim). The membranes were then processed for chemiluminescence detection of the PCR products by the Genius system according to the manufacturer's instructions (Boehringer Mannheim). The relative amounts of each PCR product for MRP and MDR1 were quantitated by densitometry and were normalized relative to the amount of PCR product for G3PDH that had been amplified in parallel reactions. The following primers were used (with expected size of the PCR product): MRP1, nucleotide (nt) 792 to 816; MRP2, nt 1062 to 1086 (285 bp); MRP3, nt 1062 to 1086; MRP4, nt 1460 to 1484 (533 bp); MDR1-1, nt 410 to 441; MDR1-2, nt 664 to 695 (286 bp); G3PDH-1, nt 75 to 100; G3PDH-2, nt 670 to 696 (621 bp). These primers were purchased from The Midland Reagent Company (Midland, TX). For MRP, either primer pair MRP1/MRP2 or MRP3/MRP4 was used with identical results. The specificity of the MRP primer pairs was confirmed by direct sequencing of the resulting PCR products, using the Circumvent sequencing kit from New England Biolabs (Beverly, MA).

Southern blotting. Total genomic DNA from paired leukemia samples was isolated by SDS lysis and proteinase K digestion, followed by phenol/chloroform extraction and ethanol precipitation. Purified DNA (10 µg) was digested with EcoRI overnight and electrophoresed on a 0.7% agarose gel. DNA from HL60 and HL60/AR cells was treated identically and provided control samples of an unamplified and an amplified MRP gene on the same blot. The DNA was denatured, transferred onto a nylon membrane (Boehringer Mannheim) and hybridized at 65°C with a PCR-generated digoxigenin-labeled DNA probe consisting of the first 890 bp of the coding region of the MRP cDNA. After hybridization the membrane was washed in 0.5 × SSC/0.1% sodium dodecyl sulfate (SDS) at 65°C, and bound probe was detected by the Genius chemiluminescence method (Boehringer Mannheim) according to manufacturer's instructions.

RESULTS

Validation of PCR assay. The yield of PCR product is proportional to the starting amount of the template only under conditions in which PCR amplification proceeds exponentially. To establish conditions under which the amount of PCR product would be directly proportional to the amount of template used, cDNA was synthesized from total RNA
from HL60 cells by reverse transcription. The cDNA was then serially diluted, and each dilution was separately amplified using specific primer pairs for MRP or G3PDH. The resulting products were separated by gel electrophoresis and transferred onto a nylon membrane. After transfer, the membranes were processed for chemiluminescence detection of the PCR products, which were quantitated by densitometry of the bands after exposure to x-ray film (Fig 1A). The relative band intensities were then plotted against the calculated amount of RNA originally present in each dilution (Fig 1B). The observation that the curves were linear and parallel with a slope of approximately 1 indicated that this method could be used to compare levels of MRP expression between different samples. Furthermore, these studies also demonstrated that a fivefold to 10-fold dilution for MRP (corresponding to 0.1 to 0.2 μg RNA) and a 50-fold to 100-fold dilution for G3PDH (corresponding to 0.01 to 0.02 μg RNA) gave amplified signals whose intensity was within the linear range of the reaction. These dilutions were used for the analysis of MRP expression in all of the samples described below.

MRP expression in normal cells. To provide a baseline for comparison, MRP expression was analyzed by quantitative RT-PCR in normal B cells, T cells, and granulocytes as well as in normal CD34+ and CD34+ bone marrow cells. Results were normalized to levels of expression of the housekeeping gene G3PDH that had been determined in parallel. HL60 human promyelocytic leukemia cells were included in each experiment to provide an internal standard. MRP mRNA levels in HL60 cells were found to be similar to the average MRP mRNA level observed in 55 different unselected human tumor cell lines (A. Fojo, personal communication, May 1993). The MRP/G3PDH mRNA level in HL60 cells was arbitrarily assigned a value of 1.0, and levels of all other samples were compared with this value. All normal lymphohematopoietic cell populations analyzed had levels of MRP mRNA that were similar to those in HL60 cells, with the exception of T cells, which had twofold higher MRP mRNA levels (Fig 2).

MRP expression in AML and ALL. Using the same approach, expression of MRP mRNA was analyzed in 43 AML (29 collected at initial diagnosis and 14 at time of disease...
relapse) and 19 ALL (15 collected at diagnosis and 4 at relapse) samples. Results were again normalized to levels of expression of the housekeeping gene G3PDH that had been determined in parallel and were compared with HL60 cells (Table 1). MRP expression in the clinical samples ranged from undetectable to 8.6-fold greater than in HL60 cells, with a mean relative expression level of 1.0 at diagnosis and 1.7 at relapse in AML (median, 0.9 and 1.5; range, 0.01 to 3.1 and 0.4 to 5.0, respectively; \( P < .01 \)) and 1.5 at diagnosis and 1.4 at relapse in ALL (median, 0.8 and 1.4; range, 0.4 to 8.6 and 1.2 to 1.5, respectively). It should be noted that MRP expression levels in ALL samples at diagnosis were distributed over a wide range, from as low as 0.4 to as high as 8.6, and that corresponding samples at relapse from the two highest expressors were not available. This resulted in artificially distorted mean values. By comparison, in the doxorubicin-selected, Pgp-negative, multidrug-resistant HL60/AR variant that is more than 100-fold resistant to doxorubicin, MRP expression was 9.5-fold higher than in the parental HL60 cell line.

There was no clear relationship between MRP expression at diagnosis and leukemia type (Fig 3A), response to therapy (Fig 3B), and duration of remission (Fig 3C). Furthermore, there was also no relationship between MRP expression and expression of the early myeloid marker CD34 on the leukemia cells (median MRP expression was 1.1 if CD34 and 1.0 if CD34), in agreement with our observation that MRP expression in CD34 and CD34 normal marrow cells was similar. In contrast, a statistically significant \( P < .01 \) increase in MRP expression was observed when samples from

**Table 1. MRP and MDR1 Expression in AML and ALL Samples**

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<th></th>
<th>AML</th>
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<th>MRP Expression*</th>
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<tr>
<td>All samples</td>
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<td>At diagnosis</td>
<td>1.0 ± 0.1 (N = 29)</td>
<td>1.5 ± 0.5 (N = 15)</td>
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<td>1.0 ± 0.3 (N = 17)</td>
<td>1.2 ± 0.7 (N = 6)</td>
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<tr>
<td>At relapse</td>
<td>1.7 ± 0.3 (N = 14)</td>
<td>1.4 ± 0.1 (N = 4)</td>
<td>1.0 ± 0.1 (N = 2)</td>
<td>0.9 ± 0.2 (N = 4)</td>
<td>1.0 ± 0.6 (N = 4)</td>
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<td>( P ) value</td>
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<tr>
<td>At diagnosis</td>
<td>1.0 ± 0.2 (N = 13)</td>
<td>0.7 ± 0.2 (N = 4)</td>
<td>1.7 ± 0.6 (N = 6)</td>
<td>1.9 ± 1.0 (N = 3)</td>
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<tr>
<td>At relapse</td>
<td>1.8 ± 0.3 (N = 13)</td>
<td>1.4 ± 0.1 (N = 4)</td>
<td>0.8 ± 0.3 (N = 6)</td>
<td>1.2 ± 0.9 (N = 3)</td>
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<td>( P ) value</td>
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MRP and MDR1 expression was determined by quantitative RT-PCR as described in Materials and Methods and normalized with G3PDH expression determined in parallel reactions. Each individual sample was analyzed at least twice, and the average of each sample was used to calculate the expression relative to that in HL60 or SW620 cells. These relative expression levels were used to calculate the means \( \pm \)SE presented in this table. For comparison, MRP expression in HL60 cells was 1.0; in HL60/AR cells, 9.5; and in SW620 cells, 1.8. MDR1 expression in HL60 and HL60/AR cells was undetectable and in SW620 cells was 1.0.

Abbreviation: NS, not significant.

* Relative to MRP expression in HL60 cells.

† Relative to MDR1 expression in SW620 cells; only samples with quantifiable amounts of MDR1 were used to calculate means.

‡ Student’s t-test.
relapsed patients were compared with samples from patients with newly diagnosed leukemia (Table 1). From a total of 29 AML and 15 ALL patients with newly diagnosed acute leukemia, paired samples (i.e., the first harvested at the time of diagnosis and the second at the time of leukemic relapse) were available from 17 (13 AML and four ALL). When MRP expression at diagnosis and recurrence was compared in each pair separately (Fig 4A), the mean increase in MRP expression at relapse was 2.8-fold in AML and 2.3-fold in ALL (median, 1.5- and 2.2-fold, respectively). Of the 17 paired samples, one (6%) showed a decrease at relapse, and two (12%) remained equal (less than 220% change). However, the vast majority of paired samples (82%) showed an increase in MRP expression at the time of relapse. In those, expression at relapse ranged from 1.2- to 15.3-fold that at the time of diagnosis (one paired sample was not quantifiable due to a lack of detection at diagnosis). In summary, MRP was detected in all but one of the leukemia samples analyzed, and there appeared to be a tendency for higher MRP expression at the time of relapse.

**MDR1 expression in AML and ALL.** Expression of MDR1 mRNA was also analyzed by quantitative RT-PCR in all 43 AML and 19 ALL samples. Expression levels were normalized to those of the housekeeping gene G3PDH and then compared with MDR1 expression in SW620 cells, a cell line that naturally expresses low levels of MDR1 (Table 1). No MDR1 expression was detected in HL60 and HL60/AR cells. In contrast, in the KG1a human leukemia cell line that had previously been shown to exhibit a twofold to threefold decrease in LD50 for daunorubicin and etoposide in the presence of Pgp modulators,16,61 MDR1 expression was 2.8-fold higher than in SW620 cells.

In 27 of the 62 clinical samples (17 of 43 AML and 10 of 19 ALL), MDR1 expression was too low to be quantitated. In the remaining 35 clinical samples, relative expression of MDR1 ranged from 0.05-fold to 3.7-fold the level expressed in SW620 cells, with a mean relative expression level of 1.1 at diagnosis and 0.9 at relapse in AML (median, 0.6 and 0.8; range, 0.05 to 3.7 and 0.2 to 2.0, respectively) and 1.2 at diagnosis and 1.0 at relapse in ALL (median, 0.9 and 0.5; range, 0.1 to 3.7 and 0.3 to 2.9, respectively). Consistent with our previous results obtained using a functional assay for Pgp,16 only six of 62 samples manifested a level of MDR1 expression that approached the level observed in KG1a cells.

There was no correlation between MDR1 expression and leukemia type (Fig 5A), response to therapy (Fig 5B), or duration of remission (data not shown). Furthermore, there was also no correlation between expression of MDR1 and MRP at diagnosis ($r = 0.3$, $P = .2$). In contrast to the results with MRP, there was also no clear pattern of change in MDR1 levels at the time of relapse (Fig 4B). Of the 17 paired samples, nine (six AML and three ALL) were informative, with a mean increase at the time of relapse of 1.1-fold in AML and 0.8-fold in ALL (median, 0.6- and 0.8-
Fig 5. Expression of MDR1 mRNA in newly diagnosed leukemia. Relative MDR1 expression was examined as a function of French-American-British classification (A) or response to initial therapy (B). MDR1 mRNA levels were determined by quantitative RT-PCR as described in Materials and Methods, were normalized for G3PDH mRNA levels, and are expressed relative to the level of MDR1 mRNA in SW620 cells. For comparison, MDR1 was undetectable in HL60 and HL60/AR cells, and expression levels in KG1a cells were 2.5-fold those in SW620 cells. KG1a cells show a 60% increase in daunorubicin accumulation in the presence of Pgp modulators. NR, no response; Dx, diagnosis; Rx, therapy.

In conclusion, MDR1 expression at relapse ranged from 0.05- to 3.5-fold that at the time of diagnosis, with one sample (11%) remaining unchanged (less than ±20% change), three (33%) increased, and five (55%) decreased. In conclusion, MDR1 expression did not appear to correlate with any of the clinical parameters analyzed. Although samples with MDR1 mRNA levels too low to be quantitated by densitometry were excluded from this analysis, the results remained qualitatively similar when relative MDR1 expression in these samples was arbitrarily set at 0.01 (1% of SW620). Thus, we do not believe that the omission of samples with very low MDR1 levels has led to an artificial distortion of the results.

Genomic DNA analysis. In several drug-selected cell lines that overexpress MRP, amplification of the MRP gene has been described (Schneider et al, unpublished data, June 1993). Therefore, we analyzed genomic DNA from five paired samples (three AML and two ALL) in which MRP mRNA levels were 1.2- to 2.1-fold higher at relapse than at diagnosis (in one pair, no MRP mRNA was detected at diagnosis, but was clearly detectable at relapse; Fig 6). For comparison, we also analyzed genomic DNA from HL60 cells and their drug-resistant derivative HL60/AR cells, which had 9.5-fold higher MRP mRNA levels. While there was clear evidence for amplification of the MRP gene in HL60/AR cells, we did not find any indication that the MRP gene is amplified in the relapsed leukemia samples.

DISCUSSION

In a previous study of marrow samples from patients with newly diagnosed and relapsed AML, we reported that drug sensitivity did not correlate with Pgp function or topoisomerase II levels. In this report these earlier studies were extended by analyzing levels of MDR1 and MRP mRNA by quantitative RT-PCR. In agreement with our previous results, there was no correlation between MDR1 expression and response to therapy. Moreover, comparison of samples obtained at the time of diagnosis and again at the time of relapse did not show any consistent changes in MDR1 expression. In contrast, MRP mRNA could be readily detected and quantitated in all but one leukemic sample. Moreover, there was a statistically significant (P < .01) increase in the average MRP expression level at the time of relapse compared with that at the time of diagnosis (Table 1). This result is in agreement with observations by Hart et al and Zhou et al who also found a slight (albeit statistically insignificant) increase in MRP expression in relapsed compared with newly diagnosed AML. Furthermore, increased MRP expression at the time of leukemic recurrence was also observed in 14 of 17 cases when we directly compared paired samples (Fig 4A). Therefore, our results are consistent with the hypothesis that increased MRP expression might contribute to leukemic relapse.

Despite the observation that MRP mRNA levels tended to increase at the time of relapse, there was no obvious correlation between pretreatment MRP mRNA levels and response to therapy or duration of remission. This lack of correlation between pretreatment mRNA levels and clinical
response could result from variations in one or more factors that might differ among different leukemic marrows, notably posttranscriptional regulation of protein expression, protein stability, and cell-to-cell heterogeneity of gene expression. Further studies at the single cell level are required to address some of these issues.

The question arises whether the moderate increases in MRP expression at relapse are sufficient to confer drug resistance. Studies in drug-selected cell lines indicate that 10- to 15-fold increases in MRP mRNA levels can result in up to 100- to 200-fold resistance to doxorubicin and 30- to 150-fold resistance to etoposide. In addition, Grant et al reported that a 50% increase in MRP RNA levels in cells transfected with an MRP expression vector resulted in a 50% inhibitory concentration that was 50% higher for doxorubicin despite the observation that protein synthesis from the transfected RNA appeared less efficient than from the endogenous RNA. Therefore, even the moderate increases in MRP mRNA levels observed in the relapsed leukemia samples might be sufficient to convey increased drug resistance. When one considers that antileukemia agents are administered at maximally tolerated doses, even this modest increase in resistance might result in diminished clinical responsiveness.

The present studies suggest that MRP gene amplification does not play a role in the increased MRP expression in clinical samples (Fig 6). Instead, other mechanisms need to be considered. For instance, it is possible that MRP was heterogeneously expressed in the original leukemic cell population and that MRP-overexpressing subclones have selectively survived the chemotherapeutic regimens used in the present study. Alternatively, it is possible that MRP expression has been directly induced by the drugs used during therapy, in much the same way that MDR1 appears to be induced in certain cell lines. Further studies on the frequency of MRP-positive cells and on the regulation of MRP gene expression are required to distinguish between these possibilities.

At present it is unknown whether our observations are widely representative of MRP expression in acute leukemia. The practical requirement that the samples in the present study contain greater than 10⁷ cells with a majority of blast cells might have resulted in a skewed population that contained a relative paucity of certain leukemic phenotypes, such as secondary leukemias and leukemias arising in the setting of an antecedent myelodysplastic syndrome. Likewise, the emphasis on paired samples might have resulted in a study that was weighted toward patients who had poor disease-free survival. Consistent with this possibility, we note that seven of the 17 patients (41%) providing paired samples never achieved a CR and that the median disease-free survival for the remaining patients providing paired samples was only 254 days. In contrast, mean duration of disease-free survival among the total patient population that had achieved CR (77%) was 420 days. Finally, the chemotherapeutic regimens used in the present study might have somehow selected for cells that preferentially expressed MRP rather than MDR1, whereas other regimens might select for cells that express MDR1. Nevertheless, further studies of MRP expression in the context of various antileukemic regimens appear warranted.

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Increased expression of the multidrug resistance-associated protein gene in relapsed acute leukemia

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