Expression of the mdr-1/P-170 Gene in Patients With Acute Lymphoblastic Leukemia

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Increased expression of the multidrug resistance gene (mdr-1/P-170) and the dihydrofolate reductase (DHFR) gene have been implicated in the development of in vitro drug resistance. Overexpression, with or without gene amplification, is seen in the development of drug resistance in culture and it has been postulated that genetic modulation of mdr-1/P-170 and DHFR may also be involved in the development of clinical drug resistance. We screened lymphoblasts from 28 patients with acute lymphoblastic leukemia (ALL) for evidence of overexpression of mdr-1/P-170 using RNAase protection. RNA in situ hybridization and immunohistochemistry. Overexpression of mdr-1/P-170 without gene amplification was detected in samples from four patients (three after multiple relapses, one at presentation). Overexpression of mdr-1/P-170 was heterogeneous within the population of malignant lymphoblasts as demonstrated by RNA in situ hybridization, immunohistochemistry, and drug uptake using daunomycin autofluorescence analysis. There was no evidence of overexpression of DHFR in any of the eight patient samples tested by RNAase protection nor was there any evidence of gene amplification in 11 patient samples on Southern blot analysis. From these observations it appears that overexpression without gene amplification of mdr-1/P-170 may be one mechanism of clinical drug resistance in ALL.

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T HE IDENTIFICATION of mechanisms of drug resistance is an important goal of current research. Emphasis has been placed on elucidation of specific mechanisms of resistance because this is viewed as the first step toward overcoming drug resistance. Two systems that have been studied extensively in vitro are multidrug resistance mediated by P-170 and methotrexate resistance secondary to increased levels of DHFR. Although these systems have been exhaustively characterized in vitro, there is very little information on their clinical prevalence.

P-170 is a 170,000 dalton membrane glycoprotein originally described by Ling that is the product of the mdr-1 gene. This glycoprotein has been demonstrated to mediate resistance in vitro to multiple structurally dissimilar drugs including the anthracyclines, actinomycin D, the vinca alkaloids, and the epipodophyllotoxins. Cross resistance to all of these drugs can occur after exposure to only one of them, although the patterns of cross resistance vary among different isolates. P-170 functions as an energy-dependent efflux pump so that a cell with a high level of mdr-1/P-170 expression can more effectively eliminate cytotoxic drugs, resulting in decreased drug accumulation. In vitro this resistance can be reversed by a group of diverse drugs that include verapamil and other calcium channel blockers, several antiarrhythmic agents, and the phenothiazines. These compounds inhibit the pump in a reversible manner, resulting in increased accumulation of the chemotherapeutic agents and enhanced cytotoxicity. In clinical samples, increased expression of the P-170 glycoprotein was first reported in two human ovarian cancer specimens assayed by immunoblotting. Since that initial report, mdr-1/P-170 expression has been reported in a variety of normal and neoplastic tissues, but its role remains unclear.

The larger body of research has been published establishing a correlation between the levels of dihydrofolate reductase (DHFR) and resistance to methotrexate. Increased levels of DHFR have been obtained in vitro in both human and animal cell lines, and this system has been invaluable in furthering an understanding of the phenomenon of gene amplification. Most cell lines exhibit the karyotypic changes of gene amplification, including minute or double minute chromosomes, abnormally banding regions, or homogeneously staining regions. But in spite of the long established in vitro results, there are few clinical examples supporting a role for DHFR overexpression or amplification in clinical methotrexate resistance.

Acute lymphoblastic leukemia (ALL), the most common malignancy of childhood, is considered a chemoresponsive malignancy, with remissions achieved in 95% of cases and long-term disease-free survival attained in 50% to 75%. Induction regimens and some salvage chemotherapy protocols use vincristine and daunomycin, both of which are members of the natural product class of antitumor agents that comprise the multidrug resistant phenotype mediated by mdr-1/P-170. In addition, methotrexate is standard therapy for the maintenance of remission and plays a role in CNS preventive therapy. Because of the importance of these agents in remission-induction and salvage treatment of ALL, and because of the role of mdr-1/P-170 and DHFR in the development of in vitro resistance to these agents, we chose to examine clinical specimens from ALL patients for evidence of overexpression or gene amplification of mdr-1/P-170 and DHFR. In this report we present those results.

MATERIALS AND METHODS

Sample collection. Following informed consent, samples were obtained from bone marrow or peripheral blood of 28 patients with a diagnosis of ALL. Patients ranged in age from 2 to 41 years of age.

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and included nine newly diagnosed patients and 19 patients at relapse. Acute leukemias confirmed at both diagnosis and relapse were defined as lymphoblastic based on French-American-British (FAB) morphology. Mononuclear cells were isolated from samples by Ficoll-Hypaque density gradient centrifugation in a modification of the method of Boyum. Cells from samples that were not analyzed fresh were frozen in the vapor phase of liquid nitrogen in RPMI-1640 with 20% fetal bovine serum and 10% dimethyl sulfoxide (DMSO) until use.

**Materials.** Deoxycytidine 5'-[α-32P] triphosphate (3,000 Ci/mmol, 1 Ci = 37 GBq), uridine 5'-[α-32P] triphosphate (3,000 Ci/mmol) and 35S Uridine 5'-[α-thio] triphosphate were obtained from New England Nuclear, Boston. Lymphocyte separation medium (LSM) was obtained from Organon Teknika Corporation, West Chester, PA. All other reagents were of the highest quality and purity available.

**Probes.** Two mdr-1 probes were used in our studies. Measurements of mdr-1 expression in the RNase protection assay used a 1 kilobase (kb) genomic sequence from the 5' end of the gene subcloned into pGEM-3 and linearized with PvuII. The fragment has previously been described by Ueda et al. The 1 kb PstI fragment contains a promoter region of the human mdr-1 gene as well as an additional 5' sequence including a putative exon (Fig 1). Transcripts originating from this promoter, referred to as the "upstream promoter," protect a 134 base pair (bp) fragment of the RNA probe. A 323 nucleotide sequence of the same probe is protected by mdr-1 transcripts that originate at an "upstream promoter." Analysis of DHFR expression by RNase protection used a 530 bp sequence of the DHFR pseudogene 1 inserted into pGEM-3 vector. β-actin expression was measured using a 412 bp cDNA fragment from the 3' untranslated region of a human β actin cDNA subcloned into pGEM-3.

Southern blot analysis was performed with a nick-translated 1.4kb fragment from the middle third of the mdr-1 gene. This fragment was also used as a template for the RNA probe used in the RNA in situ hybridization experiments.

RNA probes were synthesized according to the protocol of Melton as modified in the Promega Technical Bulletin 11. The concentrations of unlabeled uridine triphosphate (UTP) were as follows: for mdr-1, 25 μmol/L; for DHFR, 50 μmol/L, and for β-actin, 400 μmol/L.

Nick translated mdr-1 and DHFR probes were synthesized by the method of Rigby et al., adapted by the Dupont NEN Co, Wilmington, DE.

**RNase protection technique.** Total RNA was hybridized with 1 x 10^6 cpm of antisense RNA probe using a modification of the method of Melton et al. The samples were separated on a 6% polyacrylamide gel at 1,500 volts for two to three hours followed by autoradiography for one to three days.

**RNA in situ hybridization.** Lymphoblasts were thawed at 37°C, washed with phosphate-buffered saline (PBS), and resuspended in media and cytocentrifuged onto a sterile histostick-covered glass slide. Prehybridization, hybridization, washes, and autoradiography were performed as previously described.

**Immunofluorescence studies.** Immunofluorescent studies were performed using the MRK-16 monoclonal antibody (MoAb), which recognizes an extracellular determinant of P-170. An antimonocyte immunoglobulin (Ig)G linked to rhodamine allowed for visualization of the cells binding MRK-16 under fluorescent light. The lymphoblasts were also screened with HB-21, an MoAb to the transferrin receptor.

**Daunomycin uptake.** Single-cell analysis of daunomycin uptake was performed as previously described by measuring autofluorescence with a 540 nm light after a five-minute incubation of cells immobilized on a poly-L-lysine-coated dish or cytocentrifuged on a slide.

**RESULTS**

Figure 2 presents a schematic of the time course of treatment, relapse, and sample collection on each of the 28 patients studied for mdr-1/P-170 expression. We were able to isolate RNA from nine patients at the time of initial presentation, five patients evaluated at the time of first relapse occurring during or after the maintenance phase of therapy, and 15 patients after multiple relapses. This total of 29 samples for 28 patients reflects the presence of two samples obtained from the same patient (patient 2). As detailed below, four patients had increased levels of mdr-1/P-170 expression and are identified in Table 1. Three of these patients had suffered multiple relapses before the samples were obtained while the fourth had increased levels of mdr-1/P-170 at the time of diagnosis. Additional details on these four patients are summarized in Table 1. All patient samples were analyzed by RNase protection assay. In addition, several samples were analyzed more extensively as outlined below.

Expression of mdr-1/P-170 was determined using the RNase protection technique (Figs 3 and 4). The three lanes at the right of Fig 3 demonstrate the fragments of each individual probe protected by RNA. Protection of the β-actin antisense probe gives rise to various bands including a faint

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**Fig 1.** Schematic of the mdr-1 probe used in the RNase protection experiments and the size and origin of the fragments protected by transcripts originating from either of the two mdr-1 promoters.
Fig 2. Schematic of the time course of treatment, relapse, and sample collection on each of the 28 patients studied for mdr-1/P-170 expression. In addition to the patients identified by the bracket as having relapsed, three additional patients who relapsed during maintenance therapy are shown on the protocol schema at the bottom of the figure. For the patients identified by the bracket, each vertical line represents a relapse and the arrow indicates the time of sample collection. (I, induction; C, consolidation)

Band that migrates just below the 130 bp mdr-1 fragment. Protection of the DHFR antisense probe gives a predominant band of 530 bp. Two fragments can be protected by mdr-1/P-170 transcripts, a 130-bp fragment and a 323 bp fragment\(^1\) (Fig 1 and Materials and Methods section). In Fig 3, only the sample from patient 5 demonstrates increased levels of mdr-1/P-170 expression and shows protection of fragments arising from both mdr-1/P-170 promoters. The results in Fig 4 demonstrate increased expression in patients 5, 9, 10, and 13. Due to varying yield of RNA from the clinical samples, the amount of RNA loaded varied from lane to lane. In addition, because several β-actin probes all gave multiple bands that hindered interpretation of the results, this was deliberately omitted from this analysis. Therefore, results from this experiment should be considered qualitative (ie, mdr-1 expression elevated or not) rather than quantitative. For patient 13, increased expression is demonstrated even though only 3.5 µg of RNA were loaded, representing all the RNA available from this clinical sample. Two of the four patient samples (patients 5 and 9) possess transcripts originating from both promoters while the other two (patients 10 and 13) have messages that originate primarily from the upstream promoter. The last two lanes of this autoradiogram are from patients in whom mdr-1/P-170 expression is very low or absent, with no detectable message identified even when 120 µg of RNA was used in the hybridization. These two are representative of the samples with low or undetectable expression of mdr-1/P-170 and give an indication of the degree of overexpression found in the four positive samples. This figure also includes three additional positive samples: the positive control cell line KB 8-5 and two cell lines established from patients with Hodgkin's disease who had relapsed following therapy.

In addition to the mdr-1/P-170 results, we simultaneously examined some of the samples for increased expression of DHFR by RNase protection. None of the nine samples tested from patients with ALL at presentation or at relapse had any evidence of increased expression of DHFR detected by this approach.

In addition, we analyzed the samples for the presence of gene amplification. Hind III-digested DNA was examined using nick-translated mdr-1 and DHFR probes. Because of the limited supply of clinical material from the patients studied, not all patients studied for overexpression could be studied for gene amplification. None of the 11 samples probed for mdr-1 and DHFR showed any evidence of gene
amplification (data not shown). Three of the four patients with increased mdr-1 expression were included in this analysis. This finding confirms our earlier experience that overexpression of mdr-1 can occur without amplification of the gene.

A limitation of the RNase protection assay is the inability to determine the distribution of expression of a given message within a population of cells. With this approach one cannot discern if a message is expressed equally in all cells or if there is heterogeneity of expression in the population. In order to better understand the results in our clinical samples, we studied the distribution of mdr-1/P-170 overexpression in the population by examining individual cells by in situ hybridization and immunofluorescence. Figure 5 shows the results of such an analysis in one of the patients with increased levels of mdr-1/P-170 studied using three different approaches. Figure 5A shows the immunofluorescence pattern obtained with an antitransferrin receptor antibody that is used as a control to indicate viable, growing cells. Over 95% of the cells were positive with this antibody as well as with a β-actin riboprobe in RNA in situ hybridization studies (not shown). Figure 5B presents the results of an in situ hybridization with an mdr-1/P-170 antisense probe and demonstrates marked heterogeneity in the expression of mdr-1/P-170 in this population of cells. This result is in contrast to those observed with multidrug resistant cell lines selected in culture where a more homogeneous pattern of mdr-1/P-170 expression is seen (data not shown). Figures 5C and D are the phase-contrast photograph and the immunofluorescence results obtained with the anti-P-170 antibody, MRK-16. The heterogeneity seen in this experiment confirms the RNA in situ hybridization results, again demonstrating high levels in some cells and much lower amounts in others. Finally, in order to assess whether the P-170 glycoprotein expressed in these cells was functional and to determine whether P-170 antagonists such as verapamil could block its function, we examined the cells for daunomycin autofluorescence after a five-minute incubation without (Fig 5E) or with (Fig 5F) 10 µg/mL verapamil. Identical photographs were obtained to allow for a direct comparison. The pattern of fluorescence in the absence of verapamil shows the heterogeneity that would have been predicted from the RNA in situ hybridization and immunofluorescence studies. This heterogeneity is converted to a more homogeneous pattern by the addition of verapamil, resulting in accumulation of daunomycin in the vast majority of cells. Similar experiments performed with samples having low levels of mdr-1/P-170 expression showed a homogeneous pattern that was not affected by the addition of verapamil. Thus, in this ex vivo analysis of cells not selected by any tissue culture techniques, drug accumulation can be increased by the addition of a P-170 antagonist.

**DISCUSSION**

The identification of the mechanisms of drug resistance in clinical oncology remains an important goal of current research efforts. Elucidation of the mechanisms of resistance can form the basis for more rational therapy. In the case of multidrug resistance mediated by P-170, clarification of its role in clinical drug resistance is especially important because of the possibility that resistance mediated by overexpression of this glycoprotein can be overcome. In the present study, we report our investigations into possible mechanisms of drug resistance in ALL. Our results, which must be considered preliminary, indicate that the frequency of overexpression of the mdr-1 gene depends on the setting from which the sample is obtained. Our interpretation is that overexpression can play a role in mediating drug resistance in some patients with ALL, especially at the time of relapse.

In attempting to identify the molecular basis of drug resistance, we sought to obtain information that could help to answer several questions: (1) How frequently does overexpression of a putative mechanism of resistance occur and in what setting is it more likely to be found? (2) Is overexpression homogeneous or heterogeneous in the population of tumor cells? (3) At what level is overexpression found? and (4) Can the resistance be overcome in clinical samples?

To try to obtain this information, we used samples previously obtained from patients either at presentation, at the time of a first relapse, or after multiple relapses. We placed a greater emphasis on gene overexpression than gene amplification since expression of the mdr-1 gene in normal human tissues can vary several hundredfold without gene amplification.39

The first group consisted of samples obtained from patients with ALL at the time of presentation. When initially
treated, ALL is typically responsive to induction chemotherapy and one would predict that expression of mdr-1/P-170 would be absent or low in the majority of these samples. Our results support this prediction, because we found that eight of nine samples (89%) obtained from patients at the time of initial diagnosis had uniformly low levels of mdr-1/P-170. Only one of the nine samples obtained at the time of initial diagnosis had elevated mdr-1/P-170 expression, this patient failed to achieve a remission despite aggressive therapy. While this does not prove causation, we found this observation interesting.

The second group included patients who relapsed during maintenance therapy or after the completion of treatment. Maintenance therapy for these patients consisted predominantly of 6-mercaptopurine and methotrexate. Because of this, one would expect that samples from these patients would be less likely to represent natural product failures and thus should have low levels of mdr-1/P-170 expression. Only five samples were obtained from patients in this category (preventing firm conclusions), but none had evidence of mdr-1/P-170 overexpression.

The final group consisted of patients who had suffered multiple relapses. In practice, reinduction regimens often include the natural product agents that comprise the multidrug resistance phenotype. Thus, patients in this group would be more likely to have increased levels of mdr-1/P-170 expression. In our analysis, three of 15 patients (20%) in this category had elevated mdr-1/P-170 expression. Taken together, these results support a role possible for mdr-1/P-170 in some cases of drug resistance, especially in patients refractory to salvage chemotherapy. Equally important, the results also indicate that other mechanisms of...
resistance must exist. The latter is not surprising, because these patients are treated with many agents that are not part of the mdr-1 phenotype.

To further examine the distribution of mdr-1/P-170 expression on a population of lymphoblasts, we used the techniques of RNA in situ hybridization and immunohistochemistry. In the four patients who relapsed and had high levels of mdr-1/P-170 expression, several distinct populations expressing high, moderate, or no mdr-1/P-170 could be identified, confirming the results obtained in the RNAsse protection experiments (results from one patient are shown).

The finding that some cells are devoid of mdr-1 expression and could be sensitive to further therapy is an observation that is supported by the clinical finding that even in patients who have relapsed multiple times, some minor and very transient responses can often be seen. Alternatively, these cells may be resistant to the natural products by mechanisms other than overexpression of P-170.

Another important observation of our studies of individual cells was the magnitude of mdr-1/P-170 overexpression in some cells. For example, in the patient whose results are shown, the levels of expression in some cells are comparable
Fig 5. Individual cell analysis of lymphoblasts from a sample with increased mdr-1 expression. A sample from patient 5 was analyzed by several independent methods including: (A), immunofluorescence with an antitransferrin receptor antibody; (B), in situ hybridization with an mdr-1/P-170 antisense probe; (C), phase contrast photomicrograph of same field as shown in D; (D), immunofluorescence results obtained with the anti-P-170 antibody MRK-16; (E and F), daunomycin autofluorescence after a five-minute incubation without (E) and with (F) 10 μg/mL verapamil.

to those found in a multidrug resistant cell line that is 40-fold resistant to the natural products when compared with a parental cell line that expresses low mdr-1 levels and is sensitive to chemotherapy. The clinical implication of this observation is that this population of cells could prove very refractory to therapy, even dose-intensive, marrow-ablative therapy, (where two to three times the standard dose of chemotherapy is used) if the drugs used are transported by the P-170 efflux pump. Achieving a complete response in such patients would theoretically require the concomitant use of P-170 antagonists to overcome the multidrug resistant phenotype or the use of drugs not recognized by the P-170 efflux pump. Our daunomycin uptake results suggest that effective blockade of the P-170 efflux pump is possible in a clinical setting. Heterogeneity of uptake could be demonstrated in these cells with the low levels seen in cells that presumably express mdr-1. Reversibility of the phenotype by verapamil in this clinical sample is an ex vivo demonstration that the drug resistance phenotype can be reversed. Although the verapamil concentrations that are needed in a clinical setting are too toxic, other P-170 antagonists including quinidine, amiodarone, and cyclosporin are undergoing clinical trials.

Finally, it should be noted that we were unable to document gene amplification in the samples overexpressing mdr-1, a finding consistent with our previous observations in other clinical samples. Although gene amplification may occur clinically, our results suggest that this is uncommon and that overexpression must be examined at the RNA or protein levels before one concludes that a given sample is negative.

Some important questions regarding the role of mdr-1/P-170 in ALL can only be answered in a prospective study. For example, only sequential samples will provide information on the process of acquisition of drug resistance. But the most important question is whether therapeutic interventions made on the basis of detection of increased mdr-1/P-170 expression can favorably impact the outcome of the disease. This issue may be best addressed through a prospective study that uses drugs that block the P-170 efflux pump or drugs not transported by the P-170 pump in patients with high levels of mdr-1/P-170 expression. Answers to these questions could provide valuable insights not only into leukemia, but into the chemoresistance of all malignancies.

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