Expression of mdr-1 in Refractory Lymphoma: Quantitation by Polymerase Chain Reaction and Validation of the Assay

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Measurement of P-glycoprotein and the gene that encodes it, mdr-1, is an important tool for assessing the impact of multidrug resistance in clinical cancer. We evaluated mdr-1 expression by a quantitative polymerase chain reaction (PCR) assay in 78 biopsy samples from 48 patients with refractory lymphoma enrolled on a trial of infusional chemotherapy (EPOCH) in which R-verapamil was added as an antagonist of P-glycoprotein in a subset of patients whose tumors were unresponsive to treatment. Expression of mdr-1 was detectable in all biopsies at the time of enrollment on study, and a fourfold or greater increase in mdr-1 expression was noted in 42% of patients at the time of treatment failure. Expression of mdr-1 was also detectable in biopsies from patients at the time of diagnosis of lymphoma. An endogenous control gene, β2-microglobulin, was quantitated for normalization of the mdr-1 values. The use of β2-microglobulin expression for normalization was validated in a subset of samples by comparing Northern blots detecting β2-microglobulin, β-actin, and GAPDH gene expression. Immunoblot analysis suggested that no major discrepancy was present between mRNA expression and protein level. Immunophenotyping of lymphomatous lymph nodes showed that infiltration of tumor cells ranged from 8% to 95% and of normal T cells from 1% to 93%. Expression of mdr-1 in normal T cells and monocytes was also shown to be low. The mdr-1 levels in patient samples were independent of T-cell contamination, suggesting that the presence of normal cells has at best a small impact on mdr-1 measurements. Expression of mdr-1 in lymphoma can be quantitated by PCR, and wide variations in expression can be observed. Increased expression in patients with refractory disease supports an important role for Pgp in drug resistance in lymphoma. These studies will aid in the design and interpretation of clinical trials in lymphoma. This is a US government work. There are no restrictions on its use.

SINCE THE DISCOVERY of P-glycoprotein (Pgp) nearly 2 decades ago, expanding efforts have been made to determine its role in clinical drug resistance.1 These studies have included attempts at the clinical reversal of drug resistance by Pgp antagonists and the measurement of Pgp and mdr-1, the gene that encodes it, in clinical samples. Despite these efforts, the clinical significance of Pgp in most malignancies remains undetermined.2,3

In the laboratory, Pgp confers drug resistance by promoting active drug efflux from the cancer cell.4 The agents that are transported by Pgp include many of those active in lymphoma, such as doxorubicin, vincristine, and etoposide. Exposure of different tumor types to these agents in the laboratory commonly results in overexpression of Pgp and in drug resistance caused by reduced intracellular drug accumulation. Antagonists of drug efflux, such as verapamil and cyclosporine A, can reverse resistance in multidrug-resistant cell lines, although the degree of reversal depends on the level of Pgp overexpression and the presence or absence of other mechanisms of resistance.5

Although the role of Pgp in clinical drug resistance has not been clarified, increased expression of both Pgp and mdr-1 have been documented in lymphoma tissue samples in which a broad range of expression has been reported.6 This variation is partly caused by the various methods used, including immunohistochemical assays and RNA assay by Northern hybridization or slot blot analysis. The variation in the measurement of Pgp expression by immunohistochemistry in non-Hodgkin’s lymphoma, ranging from 5% to 74%, is dependent on such factors as the different antibodies and detection methods used, on the staining threshold (in percentage of cells) used to identify positive samples, and on the relative percentage of tumor and normal cells in the sample.5,7,10,11 In RNA studies, 30% to 40% of lymphoma clinical samples are reported to show mdr-1 expression.14,16

Because a number of the agents transported by Pgp are common to most lymphoma regimens, documentation of the role of Pgp in treatment failure is important. Establishing a role for Pgp in this setting would provide a basis for the conducting of trials with Pgp antagonists. In the present study, we sought to document the level and frequency of Pgp expression in tumors from patients with refractory disease enrolled in a phase II trial of infusional doxorubicin, etoposide, and vincristine with bolus cyclophosphamide and prednisone, termed EPOCH, in combination with R-verapamil, which was added as a Pgp antagonist, in a subset of patients with stable or progressive disease.17 Because samples obtained from patients are often small and serial biopsies are only practical when less invasive approaches are used, we developed a quantitative polymerase chain reaction (PCR) assay for the measurement of mdr-1 mRNA.18 In lymphoma, as with other malignancies, validation of such a methodology is important given the variable tumor load and the potential infiltration of the biopsy with normal cells that express mdr-1. We present here our results of mdr-1 expression and validation of the quantitative PCR assay in patients with malignant lymphoma.

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Patient population. From February 1990 to June 1994, biopsy samples were obtained from among 112 patients enrolled on a phase II trial of EPOCH chemotherapy and R-verapamil. In this trial, patients initially received EPOCH chemotherapy alone; if they had no regression of measurable tumor over the course of two treatment cycles or developed progressive disease, R-verapamil, a Pgp antagonist, was added to the EPOCH chemotherapy regimen on subsequent cycles. The clinical results from this trial will be reported separately. Eligible patients had relapsed or refractory low-, intermediate-, or high-grade non-Hodgkin’s lymphomas (as defined in the Working Formulation and confirmed by E.S.J.) and gave informed consent. Patients had received a median of 8 drugs and 2 prior combination chemotherapy regimens before enrollment on the study, and all patients had previously received an anthracycline-containing regimen. Pre-EPOCH biopsies were obtained a median 9 months (range, 1 to 180 months) from the last prior regimen. Post-EPOCH biopsies were obtained a median of 1 month (range, 1 to 12 months) from the last treatment with EPOCH and included 3 cohorts of patients. In cohort 1, patients with stable or progressive disease crossed over to EPOCH plus verapamil on a regular chemotherapy cycle and did undergo biopsy immediately before receiving verapamil. In cohort 2, some patients with stable or progressive disease did not go on to receive verapamil but did undergo biopsy when progressive disease was noted. In cohort 3, some patients underwent biopsy after treatment with EPOCH plus R-verapamil when progressive disease was again noted. Biopsies from patients with newly diagnosed and untreated lymphoma were obtained from the Department of Pathology, National Cancer Institute (n = 14) and from the Cooperative Human Tissues Network (n = 4).

Cell lines. mRNA isolated from SW620 cells was used as a positive control. SW620 is an unselected human colon carcinoma cell line that expresses endogenous Pgp at low levels. The cell line can be sensitized to vincristine 3- to 11-fold by the addition of verapamil. In cohort 2, some patients with stable or progressive disease did not go on to receive verapamil but did undergo biopsy when progressive disease was noted. In cohort 3, some patients underwent biopsy after treatment with EPOCH plus R-verapamil when progressive disease was again noted. Biopsies from patients with newly diagnosed and untreated lymphoma were obtained from the Department of Pathology, National Cancer Institute (n = 14) and from the Cooperative Human Tissues Network (n = 4).

T-cell and monocyte isolation. Partially purified lymphocytes and monocytes were obtained by leukapheresis of normal human volunteers and separation by counterflow centrifugal elutriation (CCE) using a Model J-6M centrifuge (Beckman Instruments, Palo Alto, CA) equipped with a JE-5.0 elutriation rotor operating at 1,725g at 20°C. Previous studies have shown the monocyte fraction to be 80% monocytes (unpublished).

RNA extraction and quantitative PCR. Tumor biopsy specimens obtained from lymphoma patients were stored frozen at −70°C, then pulverized, and solubilized in guanidinium isothiocyanate, whereas needle aspirates and cell suspensions were immediately solubilized and stored in guanidinium isothiocyanate. RNA was isolated as previously described and checked for quality by ethidium bromide staining. Aliquots of these were resuspended in guanidinium isothiocyanate (GTC) for RNA isolation and PCR performed as described below.

Northern hybridization. Northern hybridization was performed as previously described.22 To generate a riboprobe for β2-microglobulin, a 454-bp fragment of cDNA from SW620 cells was amplified by PCR with 5′ primer (1477-1504 bp) and 3′ primer (3769-3790 bp). The PCR conditions were the same as described above for the quantitative PCR except that the annealing temperature was 50°C. The PCR product was treated with Klenow Fragment and T4 kinase and subcloned into pGEM-3Z (Promega) pretreated with Sma I and calf intestinal phosphatase. The cloned vector was linearized by digestion with Kpn I (GIBCO-BRL) and a riboprobe was synthesized using the SP6 riboprobe transcription system (Promega). To detect β-actin and GAPDH expression, a 2-kb complete human β-actin cDNA (Clontech, Palo Alto, CA) and a 1.1-kb GAPDH cDNA fragment (Clontech) were labeled by random priming for Northern hybridization. Denatured radiolabeled probes (106 cpm/μL) were added to 10 mL of prewarmed Rapid-hyb buffer (Amersham, Arlington Heights, IL) and hybridization was performed at 65°C for 4 hours. After hybridization, the membrane was washed in 50 mL of 2× SSC, 0.1% (wt/vol) sodium dodecyl sulfate (SDS) for 20 minutes at 23°C and in 50 mL of 1× SSC, 0.1% (wt/vol) SDS at 65°C for 15 minutes.

Immunophenotyping. Estimations of normal T-cell, normal B-cell, and tumor cell involvement were made in 72 of the 78 samples. Immunophenotyping was performed on frozen or paraffin sections or cytospin preparations using avidin-biotin complex method, as previously described.26 Whole blood or cell suspensions were analyzed by flow cytometry. Antibodies appropriate for each method were selected from the panels shown in Table 1 and were purchased from Becton Dickinson (San Jose, CA), Dako (Carpinteria, CA), Kallestad (Austin, TX), and Biosource International (Camarillo, CA). Estimates of the percentage of T cells and B cells were determined by visual inspection in immunostains and were correlated with cell morphology to determine if the stained cells were neoplastic or reactive. For flow cytometric analysis, cells were incubated with the antibodies for 20 minutes at 23°C, as previously described.27
Sufficient RNA was obtained to allow \( mdr-1 \) quantitation by PCR in 78 patient samples. To confirm that the biopsy samples contained tumor tissue, routine histologic as well as immunopathologic evaluation was performed and the relative percentage of monoclonal and polyclonal B or T cells was assessed. Immunophenotypic determinations were made by flow cytometry or immunochemical staining, depending on the amount of tissue available. The percentage of tumor cells ranged from 8% to 95%; 68 of 72 assessable samples (94%) contained \( \geq 30\% \) tumor cells, whereas 56 of the samples (78%) contained \( \geq 60\% \) tumor cells. Biopsy samples in which the tumor cell number was not assessed (\( n = 6 \)) and those containing less than 30% tumor cells (\( n = 4 \)) were excluded from the tabulation of \( mdr-1 \) levels.

Expression of \( mdr-1 \) was determined as previously described by measuring the PCR product after amplification of serially diluted cDNA.\(^{18} \) Serially diluted \( \beta_2\)-microglobulin cDNA was amplified in parallel and both PCR products were quantitated using densitometry. Both PCR products were assigned numerical values by comparison to the expression in a control cell line, SW620, for which the PCR assay was performed with every experiment. By assigning SW620 cells an \( mdr-1 \) value of 10 and \( \beta_2\)-microglobulin a level of 1, the results in patient samples could be standardized. Once the results were standardized, a final value could be obtained. Without selection, SW620 cells maintain stable low \( mdr-1 \) levels that are considerably lower than those found in selected cell lines in vitro and are in the range observed in clinical samples. (For comparison, we assayed frequently used cell lines by this quantitative PCR methodology. Values for \( mdr-1 \) [after normalization to \( \beta_2\)-microglobulin expression] were KB 3-1, 0.04; KB 8-5, 108; KB 8-5-11, 410; KBV-1, 5,654; 8226/S, parental, 0.0; 8226/dox 6, 401; 8226/dox 40, 1823.)\(^{30,31} \) This approach to quantitation of \( mdr-1 \) is shown in Figs 1 and 2 for serial samples obtained before and after EPOCH chemotherapy in one of the patients on study. As shown in Fig 1, the quality of the RNA was first evaluated by ethidium bromide staining of a formamide gel (upper panel). One microgram of total RNA was subjected to reverse transcription with both \( mdr-1 \) and \( \beta_2\)-microglobulin 3′ primers to generate both cDNAs. The cDNAs were then serially diluted for the PCR reaction to ensure that amplification occurred within the exponential range. This step is crucial, because quantitation is only accurate when the products are obtained from the exponential phase of the reaction and not after the generation of PCR products has reached plateau.\(^{32-34} \) Plateau is thought to be due to an excess of PCR products and diminished access to primers. Depending on the level of \( mdr-1 \) expression, different dilutions of cDNA are necessary to be in the exponential range. The ng RNA shown in Fig 1 refers to the amount of RNA that generated the cDNA used in the PCR. The PCR products were separated by gel electrophoresis, stained with ethidium bromide, and then photographed on negative film for densitometry.

Figure 2 shows the results of the densitometric analysis of the PCR products as a function of the quantity in nanograms of input RNA. Numerical values for \( mdr-1 \) for the patient sample are obtained by dividing the densitometric result for the \( mdr-1 \) PCR product in an exponential portion of the curve by the densitometric result obtained for the \( mdr-1 \) PCR product arising from the same amount of input RNA from SW620 and multiplying by 10. Likewise, \( \beta_2\)-microglobulin results from an exponential portion of the curve are compared with the results obtained with \( \beta_2\)-microglobulin for SW620, which is assigned a value of 1. The \( mdr-1 \) value

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**Table 1. Antibodies Used in Immunophenotyping**

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<thead>
<tr>
<th>T-cell antibodies</th>
<th>Monoclonal Leu 1 (CD5)††</th>
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<tr>
<td>Polyvalent anti-CD3*</td>
<td>Leu 2a (CY8)††</td>
</tr>
<tr>
<td>UCHL-1 (CD45 RO)*</td>
<td>Leu 3e (CD4)††</td>
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<tr>
<td>Monoclonal</td>
<td>Leu 4 (CD3)††</td>
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<td>Leu 5 (CD2)††</td>
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<td>Leu 9 (CD7)††</td>
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<tr>
<td>B-cell antibodies</td>
<td>Polyvalent anti-light chain††</td>
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<td>Monoclonal anti-light chain§</td>
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<td>Leu 20 (CD20)*§</td>
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<td>Leu 12 (CD19)††</td>
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<td>CALLA (CD10)††</td>
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* Paraffin sections.
†† Frozen sections.
§ FITC- or PE-labeled antibodies were used in flow cytometry.
§§ Cytospins.
is normalized to the β2-microglobulin value, and a final number is obtained. The numbers for the 2 patient samples obtained before and after normalization to β2-microglobulin are plotted on the bar graph in Fig 2B.

Although β2-microglobulin is frequently used as a control gene, the observation that serum β2-microglobulin levels vary in patients with lymphoma and serve as a prognostic factor prompted an evaluation of its reliability as a control for mdr-1 expression in lymphoma. Using Northern analysis, a more traditional approach for quantitating mRNA levels, the expression of β2-microglobulin, β-actin, and GAPDHα was compared in 10 patient samples and in SW620 cells. These 10 patient samples were chosen because sufficient RNA was available to carefully measure and verify the quantity. Two micrograms of total RNA was loaded on a 6% formaldehyde/1% agarose gel. The ethidium bromide-stained RNA gel and the autoradiograms from the three Northern analyses are shown on the left in Fig 3. Using densitometry, the results obtained for the 10 samples were averaged to give a mean value for each analysis, including a mean 28S RNA value from the negative photograph of the ethidium bromide-stained RNA gel. In the graphs on the right, each mean value is plotted on the y-axis along with the variation of each of the 10 samples from the mean. The most consistent results were obtained when the value of the 28S RNA stain is used. Of the three control genes, β2-microglobulin is the most consistent, whereas β-actin shows the greatest variation.

Figure 3 also shows that the expression of β2-microglobulin is lower in SW620 cells than in the patient samples (compare the expression in 6 μg SW620, shown enclosed by the box, with 2 μg of RNA from the patient samples). Using PCR studies, we calculated this difference in basal expression to be fourfold and consequently divided all β2-microglobulin values in patient samples by 4 before normalization. Thus, having validated the PCR methodology and established the reliability of β2-microglobulin as an internal control, we relied on expression of the β2-microglobulin gene in subsequent analyses.

Figure 4 depicts the mdr-1 expression in biopsy samples with tumor cell content ≥30% obtained before and after EPOCH chemotherapy. For comparison, results from lymphoma samples from patients newly diagnosed and untreated are also shown. As in Fig 2, levels were related to the value 10, the level assigned in SW620 cells. Figure 4A depicts 18 samples obtained from patients at diagnosis and 68 samples obtained before or after EPOCH chemotherapy from patients enrolled in the study. Pre-EPOCH biopsy results are reported for 38 patients. Post-EPOCH biopsies came from patients with progressive or stable disease after treatment with EPOCH alone (n = 20) or after treatment with EPOCH plus R-verapamil (n = 10). In general, the levels of mdr-1 expression were less than 10 at the time of diagnosis (median, 1.0; range, 0.01 to 7.24) and before treatment with EPOCH (median, 2.2; range, 0.01 to 10.6). Higher levels were found after EPOCH alone (median, 8.0; range, 0.2 to 162) or after treatment with EPOCH plus R-verapamil (median, 4.0; range, 0.82 to 326). Figure 4B depicts serial samples obtained before and after EPOCH alone in 19 patients. Forty-two percent (8/19) of the patients had a fourfold or greater increase in mdr-1 levels after EPOCH chemotherapy. These results clearly show that mdr-1 expression can
increase in patients with lymphoma after treatment with natural products and also show that treatment with R-verapamil does not prevent overexpression of mdr-1.

Because the levels before EPOCH were also measured after treatment with Pgp substrates on other protocols, we sought to confirm that the low levels were reflective of the protein level. We also evaluated the contribution of admixed normal cells to the measured levels. To determine whether the low mdr-1 levels reflected low protein levels, a subset of samples in which sufficient tissue remained after RNA
isolation was examined by immunoblotting. Results are shown in Fig 5. Pgp was barely detectable in SW620 (seen on longer exposures) and undetectable in the rest of the tumors, except for 2 biopsy samples with a high protein level obtained from 2 sites from 1 patient who had progressed after treatment with EPOCH plus R-verapamil and whose tumor expressed the highest level of mdr-1, i.e., 326, in the study. Thus, Pgp was undetectable in the samples with low mdr-1 levels.

To determine the effect of normal cells present in biopsy samples, mdr-1 expression was measured by PCR in normal T cells and monocytes from 5 normal volunteers. Monocytes and lymphocytes were evaluated for expression of mdr-1 and $\beta_2$-microglobulin, as shown in Fig 6. Expression in both cell types (in peripheral blood lymphocytes, of which 90% are normally T cells, and in monocytes) was less than 10. This result suggests that contamination of the tumor sample with T cells and monocytes will not increase the level of...
EXPRESSION OF \textit{MDR-1} IN REFRACTORY LYMPHOMA

To confirm that the measured \textit{mdr-1} levels accurately reflected the level of Pgp expression in the tumor cells, immunoblot analysis was performed on a subset of samples. Pgp was undetectable in the samples predicted to have low levels on the basis of \textit{mdr-1} expression. Expression of \textit{mdr-1} was independent of T-cell content and exceeded the level present in circulating T and B cells. This study provides unequivocal evidence that increased \textit{mdr-1} expression can occur in patients with lymphoma and suggests that this may be an important mechanism of drug resistance in a subset of patients with refractory disease. It confirms and extends observations made in a previous trial at the University of Arizona in which the investigators attempted a reversal of Pgp-mediated clinical resistance in non-Hodgkin’s lymphoma. In that study, high-dose infusional verapamil was added to the CVAD chemotherapy regimen administered by continuous infusion over 4 days. The response rate was 72% and immunohistochemical studies demonstrated Pgp expression in 1 of 39 previously untreated and in 5 of 9 drug-resistant patients.

\textit{mdr-1} expression above 10 but could dilute higher levels of expression in tumor cells. Thus, we decided that samples in which tumor cells constituted less than 30% of the sample would be excluded from analysis. Indirect evidence that the effect of T cells is negligible is found in Fig 7, in which the percentage of T cells is plotted against the \textit{mdr-1} expression level. For Fig 7, the 4 patient samples that contained less than 30% tumor cells were included. Those 4 had percentages of T cells and \textit{mdr-1} values of 83% and 7.6, 75% and 5.5, 55% and 42.6, and 75% and 15, respectively. As shown, there is no evidence that T-cell content affects \textit{mdr-1} expression.

\textbf{DISCUSSION}

The studies presented here were designed to quantify the expression of \textit{mdr-1} in patients with refractory lymphoma before and after treatment with natural product chemotherapy and to validate the PCR assay used in these measurements. RNA was harvested from biopsy samples obtained from patients enrolled in the Medicine Branch phase II trial of infusional EPOCH chemotherapy. \textsuperscript{17} The expression of \textit{mdr-1} was measured using a quantitative PCR assay and was normalized to the level of expression of an endogenous gene, \textit{\beta_2}-microglobulin, to control for RNA loading. Comparison to the \textit{mdr-1} expression in a control cell line, SW620, allowed assignment of a value to the result. Expression of \textit{mdr-1} was detectable in all patient samples at the time of enrollment, before receiving EPOCH chemotherapy, albeit at low levels. Assay of serial samples showed that, in 42% of patients, \textit{mdr-1} expression increased at least fourfold after treatment with natural products. To confirm that the measured \textit{mdr-1} levels accurately reflected the level of Pgp expression in the tumor cells, immunoblot analysis was performed on a subset of samples. Pgp was undetectable in the samples predicted to have low levels on the basis of \textit{mdr-1} expression. Expression of \textit{mdr-1} was independent of T-cell content and exceeded the level present in circulating T and B cells. This study provides unequivocal evidence that increased \textit{mdr-1} expression can occur in patients with lymphoma and suggests that this may be an important mechanism of drug resistance in a subset of patients with refractory disease. It confirms and extends observations made in a previous trial at the University of Arizona in which the investigators attempted a reversal of Pgp-mediated clinical resistance in non-Hodgkin’s lymphoma. In that study, high-dose infusional verapamil was added to the CVAD chemotherapy regimen administered by continuous infusion over 4 days. The response rate was 72% and immunohistochemical studies demonstrated Pgp expression in 1 of 39 previously untreated and in 5 of 9 drug-resistant patients.

\textbf{Fig 5.} Immunoblot analysis of Pgp in tumor samples in 12 patients. The level of \textit{mdr-1} expression for each sample is plotted in the bar graph above the immunoblot. Total protein was harvested from samples in which additional tissue was available. Seventy-five micrograms of total protein was loaded on a 7.5% polyacrylamide gel, electrophoresed, transferred, and immunoblotted with 4007 antibody.\textsuperscript{39}

\textbf{Fig 6.} Quantitative PCR analysis of monocytes and lymphocytes. Five peripheral blood samples were collected; monocytes and lymphocytes were separated as described in the Materials and Methods. Quantitative PCR was performed (A). \textit{mdr-1} expression was measured and normalized to \textit{\beta_2}-microglobulin expression and plotted as shown (B).
The levels observed in the lymphoma biopsy samples obtained at the time of diagnosis were all less than 10, the level assigned to the control SW620 cell line. Among the pre-EPOCH biopsy samples, the median level was 2.2, and only 5 of 38 patients had levels that exceeded 5. Whether this level can confer Pgp-mediated resistance is unknown; cell lines selected in vitro for resistance have much higher levels. Because the unselected SW620 cell line can be sensitized to vincristine 3- to 11-fold by verapamil, an argument can be made that a level near 10 can confer clinical resistance. The fact that most of the patients receiving infusional EPOCH alone responded lends support to the hypothesis that levels much less than 10 do not confer Pgp-mediated clinical resistance.

Because increased levels were found after treatment with EPOCH, it is not clear why higher levels were not present before therapy because all of the patients were previously treated with drugs known to induce Pgp in vitro. It is possible that mdr-1 expression undergoes acute regulation, with induction during drug exposure and a decline over time with drug withdrawal. Consistent with this possibility, the 30 post-EPOCH biopsy samples were obtained a median of 1 month after treatment with EPOCH, whereas the 38 pre-EPOCH biopsies were obtained a median of 9 months after the most recent prior therapy. In contrast, the drug-resistant group from the University of Arizona study had received treatment within 3 months and thus more closely resembles our post-EPOCH group.

Further support for the hypothesis that mdr-1 expression undergoes regulation was obtained from studies of allelic polymorphism in mdr-1 expression (Mickley et al, unpublished observations). Among 30 patients shown to be heterozygous at the DNA level, 14 expressed only one allele at the mRNA level. Expression of only one allele is observed in vitro only in drug-selected cell lines. These results suggest that selection has occurred in these patients, resulting in expression of a single allele mdr-1 gene expression in some patients, regardless of the absolute level of mdr-1 expression.

A second possibility for the low level of expression is tumor heterogeneity, in which a variable faction of the malignant cells would overexpress mdr-1. This possibility was examined early in the present study by immunohistochemical analysis for Pgp expression and by RNA in situ hybridization for mdr-1 mRNA (data not shown). Expression was typically undetectable or nonspecific; when it was detectable, expression was homogeneous. These results suggested that the PCR approach would be more quantitative and reliable, although the chief disadvantage of PCR or any assay using total RNA is that it cannot address the problem of tumor heterogeneity.

Another potential explanation for the low levels of mdr-1 in tumor samples is a result of infiltration of tumor cells by normal lymphocytes that express lower levels of mdr-1. To evaluate this possibility, mdr-1 expression was measured by PCR in normal peripheral blood monocytes and lymphocytes. Peripheral blood lymphocytes are 85% to 90% T cells, which have been previously shown to efflux rhodamine and to express mdr-1. Levels in these cells were less than 10, as found in the patient samples. In peripheral monocytes, the mdr-1 level was less than 1, a finding consistent with previous studies reporting absence of expression in tissue macrophages and peripheral blood monocytes. Thus, normal elements could falsely lower mdr-1 levels in tumor samples by dilution of RNA. The admixture of tumor cells and normal cells also has the potential to give false-positive results. Low but detectable levels of mdr-1 could reflect expression by normal cells, whereas expression in the malignant cells could be absent. However, this possibility is unlikely in view of the absence of a correlation between increasing T-cell contamination in tumor samples and mdr-1 expression (Fig 7). Rather, both the normal and tumor cells appear to have a low level of mdr-1 expression in most cases. Similar results were reported in chronic lymphocytic leukemia, in which 16 of 34 patients had mdr-1 expression below the level found in normal lymphocytes.

The approach that we used to determine mdr-1 expression included normalization of the result in each patient sample to the level of expression of an endogenous control, β2-microglobulin. This has been used by us and other investigators as a control for RNA loading. However, reports suggesting variation in β2-microglobulin serum levels in patients with lymphoma led us to question whether it was a valid control. Comparison with β-actin and GAPDH by Northern analysis in 10 comparably loaded RNA samples showed that, although no control is perfect, β2-microglobulin was the most accurate of the control genes for the studies in lymphoma. This finding is consistent with results obtained in the 60 cell lines of the NCI drug screen, which showed the adequacy of β2-microglobulin expression as an internal control. Although ethidium staining of ribosomal RNA is most accurate, amplification of β2-microglobulin allows quantitation when there is insufficient RNA to confirm the results by gel electrophoresis and allows correction for the amount of RNA aliquoted for the reverse transcription reaction.
Measurement of mdr-1 levels is important to the analysis of clinical trials testing drug resistance reversal. Failure to see a treatment response after the addition of a Pgp antagonist may be due to use of an antineoplastic agent that is not a good Pgp substrate, inadequate levels of an antagonist, levels of Pgp that are too high to reverse or too low to be significant, or the presence of other mechanisms of resistance. Accurate measurement of mdr-1/Pgp will help distinguish between these possibilities. For example, List et al. and Marie et al. have independently reported that patients with progressive AML after treatment with natural product chemotherapy plus cyclosporine A had a loss of mdr-1/Pgp expression in serial samples of leukemic cells. These results showed that Pgp was lost as a mechanism of resistance and that other mechanisms emerged after treatment with cyclosporine as a Pgp antagonist. In our study, R-verapamil did not prevent high levels of mdr-1 expression in 3 of 10 patients (Fig 4A), which suggests that levels of the reversing agent were not sufficient to overcome Pgp-mediated resistance in those patients. It should also be noted that, whereas mdr-1 levels increased fourfold in 42% of patients, consistent with Pgp as a mechanism of drug resistance in that subset of patients, expression did not increase in 58% of patients and remained at low levels. In these patients, other mechanisms of resistance must dominate, and a clinical response to the addition of a Pgp antagonist could not be expected.

In summary, mdr-1 levels were detectable in all patients with lymphoma enrolled on study. The PCR assay allowed detection and quantitation of mdr-1 in samples that were often too small to allow accurate measurement of the RNA concentration. β2-microglobulin served as an endogenous control and was shown to be expressed at relatively constant levels among samples in which consistent RNA loading could be documented by gel electrophoresis and ethidium bromide staining. The range of T-cell contamination (from 1% to 83%) and of tumor (from 8% to 95%) noted in the lymphoma tumor samples showed the need for determining the tumor content of any patient samples. Increased expression in 42% of patients with disease refractory to EPOCH therapy indicates that Pgp has a role in drug resistance in lymphoma and that therapy may be improved by the addition of Pgp antagonists to a multidrug regimen. Studies of mdr-1/Pgp expression are critical to the interpretation of clinical trials attempting reversal of multidrug resistance.

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