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Activity of P-Glycoprotein in B-Cell Chronic Lymphocytic Leukemia Determined by a Flow Cytometric Assay

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Background: **Chemoresistance in some hematologic malignancies has been associated with overexpression of P-glycoprotein, which is encoded by the MDR1 gene (also known as PGY1). However, inconsistencies in data on frequency and clinical relevance of multidrug resistance in B-cell chronic lymphocytic leukemia (B-CLL) may reflect a need for improved techniques to detect this overexpression.** ***Purpose:*** **Our purpose was to measure P-glycoprotein activity in peripheral blood cells of B-CLL patients and to analyze possible clinical correlations (disease duration, prior treatment, Rai disease stage, lymphocyte counts, and disease progression).** ***Methods:*** **P-glycoprotein activity was assayed in peripheral blood cells of 42 consecutive B-CLL patients (22 treated and 20 untreated). We used dual fluorescence in a flow cytometric assay that detects efflux of the fluorescent dye rhodamine 123, which is transported from the cell by the P-glyprotein pump. Leukemia cells were co-stained with monoclonal antibody Leu12/CD19, and rhodamine 123 efflux was measured. Expression of MDR1 and MDR3 (also known as PGY3) messenger RNA (mRNA) was quantitatively evaluated by polymerase chain reaction (PCR) in 26 cases.** ***Results:*** **Marked rhodamine 123 efflux was observed in 34 (81%) of the 42 cases and was abolished in the presence of multidrug resistance inhibitors. Rhodamine 123 efflux was not associated with Rai stage, lymphocyte counts, duration of disease, or disease progression. Although rhodamine 123-negative cases were about equally distributed among untreated and previously treated patients, the percentage of cells with rhodamine 123 efflux was significantly lower for untreated patients than for those treated with chemotherapy regimens including at least one multidrug resistance-associated drug. MDR1 mRNA was detected in 25 of 26 cases and MDR3 mRNA in all 26. MDR1 mRNA expression was significantly correlated with rhodamine 123 efflux, whereas MDR3 mRNA expression was not significantly correlated; MDR1 and MDR3 mRNA expression was not significantly associated with Rai stage, prior treatment, or disease progression.** ***Conclusions:*** **These findings suggest that P-glycoprotein overexpression in B-CLL is intrinsic rather than acquired and that P-glycoprotein activity is enhanced after exposure to multidrug resistance-associated drugs. This enhanced activity does not seem to be associated with more aggressive disease. Our results also indicate that an assay of P-glycoprotein function combined with PCR is suitable for clinical multidrug resistance screening.** ***Implications:*** **Additional studies are needed to determine whether functional activity of P-glycoprotein, measured by rhodamine 123 efflux, is directly related to clinical drug resistance. [J Natl Cancer Inst 85:1751–1758, 1993]**

Classical multidrug resistance is characterized by overexpression of the MDR1 gene (also known as PGY1) that encodes for P-glycoprotein, a membrane efflux pump that decreases the intracellular accumulation of a variety of apparently unrelated anticancer drugs (*1,2*). Overexpression of P-glycoprotein has been reported to be

*See "Notes" section following "References."

directly associated with chemoresistance in various hematologic malignancies, such as acute myeloid leukemia and myeloma (*3-5*). Because of the highly variable clinical course of B-cell chronic lymphocytic leukemia (B-CLL), interest has been focused on the determination of the biological role of multidrug resistance expression in this disease.

Some studies (*6-8*), however, have provided inconsistent data concerning the frequency of P-glycoprotein expression in B-CLL, which has ranged from 18% to 100%. This discrepancy may reflect problems of sensitivity and specificity of the different methods used for the detection of P-glycoprotein–expressing cells.

Common techniques by which P-glycoprotein or messenger RNA (mRNA) from the encoding MDR1 gene can be examined include P-glycoprotein binding by labeled monoclonal antibodies (MAbs) such as MRK16, C219, and JSB-1; Northern blot and slot blot analyses of mRNA; and polymerase chain reaction (PCR) following synthesis of the complementary DNA (cDNA). Recently, Herzog et al. (*9*) compared different techniques for the detection of even low levels of MDR1 mRNA and suggested that PCR was the most reliable technique. Nevertheless, when one quantifies MDR1 mRNA by PCR, tumor specimens can be contaminated by nonmalignant cells expressing multidrug resistance (e.g., stromal cells) (*10*). Similarly, when MDR1/P-glycoprotein expression is measured in clinical trials, it is important to consider that post-transcription phosphorylation of P-glycoprotein may influence its function (*11,12*). Thus, there is still a demand for additional multidrug resistance-screening techniques, especially techniques that detect P-glycoprotein activity.

In the present study, we investigated P-glycoprotein activity in 42 consecutive B-CLL patients by means of a flow cytometric assay. This assay is based on efflux of the fluorescent dye rhodamine 123, which is transported by the transmembrane pump P-glycoprotein (*13,14*). The results of this assay of rhodamine transport were correlated with the expression of MDR1 mRNA, which was measured using a quantitative PCR-based technique. In addition, MDR3 (also known as PGY3) mRNA expression was measured by PCR, since study findings have suggested that the Mdr3 protein also represents a drug efflux pump (*8*).

Patients and Methods

Patients

Forty-two consecutive patients (29 males and 13 females) who had already been diagnosed with B-CLL were included. The diagnosis of B-CLL was based on peripheral blood counts (with at least 60% lymphocytes in the differential count), bone marrow smears, bone marrow biopsies, and immunophenotypes ($CD19^+$, $CD5^+$, $CD23^+$, $SmIg^+$, $HLA\text{-}DR^+$). The patients ranged in age from 42 to 82 years, with a median of 66 and a mean of 66 years.

At the time of the study, the disease stages in all patients were classified according to the prognostic staging system of Rai et al. (*15*). Furthermore, disease activity (i.e., stable or progressive disease) was defined by changes in the absolute lymphocyte count, lymph node size, marrow infiltration, and Rai stage as described elsewhere (*16*). Disease duration was defined as time from diagnosis to flow cytometric analysis. The median duration was 46 months (range, 2-151 months).

At the time of the study, 20 patients were untreated; all other patients had received cytotoxic therapy, including the combination of chlorambucil and prednisone (one patient), cyclophosphamide, vincristine, and prednisone (15 patients), or mitoxantrone and prednimustine (six patients).

Twelve patients were studied at different intervals. The clinical characteristics of all 42 patients are summarized in Table 1.

Cell Samples and Rhodamine 123 Efflux Assay

Peripheral blood samples from 42 patients were collected in 10-mL tubes containing heparin as an anticoagulant. Mononuclear cells were isolated by density-gradient centrifugation with Lymphoprep (Nycomed AS, Oslo, Norway) and washed three times in Dulbecco's modified Eagle medium buffered with 25 m*M* 3-(*N*-morpholino)propanesulfonic acid. All samples were processed within 4 hours.

For rhodamine 123 efflux studies, aliquots of a 500-μL cell suspension (10^6 cells) were incubated simultaneously with 150 ng/mL rhodamine 123 and with the phycoerythrin-labeled, B-cell–directed MAb Leu12/CD19 (Becton Dickinson, Mountain View, Calif.) for 45 minutes at room temperature. After two washes in ice-cold medium, the cells were resuspended in rhodamine 123-free medium with or without a multidrug resistance inhibitor—10 μmol/L verapamil or 1 μmol/L dexniguldipine (Byk Gulden, Konstanz, Federal Republic of Germany [FRG]). The rhodamine 123 efflux or retention was selectively determined for the phycoerythrin-labeled ($CD19^+$) cells at several intervals (up to 3 hours). Between measurements, cells were kept at 37 °C.

Flow Cytometric Analysis

Stained cells were analyzed on a FACStar flow cytometer (Becton Dickinson) equipped with a 5-W argon-ion laser emitting at 488 nm with 250-mW power. Rhodamine 123 fluorescence was collected through a 530/30-nm band-pass filter, and phycoerythrin fluorescence was collected through a 585/40-nm band-pass filter. Fluorescence gates were placed around the CD19-labeled cells, and time-dependent rhodamine 123 efflux was selectively assessed in these cells. To determine the fraction of cells showing rhodamine 123 efflux, we set a marker to the left of the fluorescence peak of cells, which were coincubated with 10 μmol/L verapamil and/or 1 μmol/L dexniguldipine. Samples were scored as negative when the fraction of cells to the left of the marker was less than 10% after a 120-minute incubation. The overlapping emission spectrum between the rhodamine 123 and the phycoerythrin signal was adequately compensated for by decreasing the voltage of the photomultiplier tube recording the phycoerythrin signal (*17*). Data were processed with Consort 30 software (Becton Dickinson). All flow cytometric studies were carried out in duplicate.

MDR1 and MDR3 mRNA Analyses by PCR

MDR1 and MDR3 mRNA analyses were performed on blood samples from 26 B-CLL patients. Total cellular RNA was isolated using guanidinium isothiocyanate, and cDNA was synthesized with 500 ng of total cellular RNA and 5 pmol/μL hexanucleotide primer (Boehringer Mannheim, Mannheim, FRG) at 37 °C for 1 hour in a volume of 50 μL 50 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 8 m*M* $MgCl_2$, 100 μg/mL bovine serum albumin, 20 m*M* β-mercapthoethanol, 1 m*M* of each deoxyribonucleotide, 1.25 U RNasin (Promega, Madison, Wis.), and 120 U Moloney murine leukemia virus reverse transcriptase (Promega). PCR was carried out using 2 μL cDNA (corresponding to 20 ng of total RNA) in a volume of 50 μL containing 50 m*M* KCl, 10 m*M* Tris–HCl (pH 9.0), 0.1% Triton X-100, 0.2 m*M* of each deoxynucleotide triphosphate, 1.5 m*M* $MgCl_2$, and 3 U Taq DNA Polymerase (Promega). The primers for MDR1 and β_2-microglobulin were exactly as described by Noonan et al. (*18*). The sequences of the primers for MDR3 were as follows: 2061-2083 (5′-TGTCAGAAGAGCCTTGATGTGG-3′) and 2193-2215 (5′-TGGCAATGGCACATACTGTTCC-3′). Thirty cycles were performed, with a denaturation temperature of 94 °C for 35 seconds, an annealing temperature of 57 °C for 30 seconds, and an extension temperature of 73 °C for 1 minute. Starting with cycle 16, the time for synthesis was extended (5 seconds per cycle). The reaction products were controlled on a 10% polyacrylamide gel that was stained with

Table 1. Clinical characteristics of all B-CLL patients, together with assay results

Patient No.	Age, y/sex	Rai stage	Prior treatment*	Lymphocyte count, $\times 10^9$/L	% B lymphocytes in peripheral blood	% rhodamine 123-effluxing cells	MDR1 mRNA levels, %†	MDR3 mRNA levels, %†
1	71/M	III	COP	7.1	61	26	1.9	77.8
2	72/F	IV	COP	32.8	91	0	0	45.2
3	82/M	III	None	18.3	92	15	4.0	92.8
4	77/F	II	NoSte	3.2	60	50	9.3	14.9
5	42/F	II	COP	63.5	92	70	24.1	127.4
6	72/M	II	NoSte	7.9	81	0	3.6	54.0
7	69/M	II	COP	24.1	68	58	9.5	83.6
8	66/M	II	COP	34.7	74	20	2.6	99.6
9	56/M	IV	COP	64.7	94	45	8.2	81.1
10	82/M	II	COP	3.3	61	28	6.2	0.9
11	75/M	I	None	15.3	65	15	12.4	0.8
12	72/F	III	NoSte	106.4	95	32	5.6	44.7
13	58/M	I	None	21.0	88	10	5.0	22.6
14	65/F	0	None	14.2	68	48	10.5	15.1
15	57/F	II	NoSte	80.7	95	25	7.8	92.6
16	72/F	III	COP	103.7	96	32	6.9	75.8
17	52/M	II	COP	13.1	65	61	25.8	74.2
18	53/M	I	COP	4.9	63	15	1.8	23.1
19	73/M	I	None	15.8	80	16	7.1	31.4
20	52/M	II	None	40.2	80	0	4.8	45.2
21	59/F	I	None	20.4	84	19	4.7	36.9
22	69/M	II	NoSte	27.1	86	42	14.8	25.5
23	61/M	I	None	8.6	60	58	10.1	14.7
24	66/F	I	None	19.9	84	10	9.6	11.0
25	73/F	II	COP	38.4	92	30	11.8	30.9
26	77/M	I	None	17.6	87	0	6.2	57.9
27	75/F	0	None	65.6	87	45		
28	65/M	I	None	28.1	75	43		
29	62/M	IV	COP	4.0	65	0		
30	66/M	II	COP	9.4	66	26		
31	74/M	II	COP	18.9	91	19		
32	59/M	IV	None	23.1	77	0		
33	63/M	II	None	74.2	81	0		
34	67/F	II	None	149.9	98	24		
35	58/M	II	None	178.5	97	36		
36	71/M	0	None	17.1	82	49		
37	59/M	II	None	52.4	70	45		
38	43/M	0	None	17.0	85	49		
39	64/M	IV	COP	10.3	67	0		
40	64/F	II	Chlor/Pred	78.3	94	17		
41	79/M	0	None	6.1	64	13		
42	69/M	II	NoSte	2.9	61	52		

*Chlor/Pred = chlorambucil + prednisone; COP = cyclophosphamide + vincristine + prednisone; NoSte = mitoxantrone + prednimustine.
†Expressed relative (%) to the β-microglobulin amplification product.

ethidium bromide and photographed. For the expression of MDR1 mRNA, the drug-sensitive cell line KB-3-1 and its drug-resistant mutant KB-8-5 (provided by Dr. M. Gottesman, National Cancer Institute, Bethesda, Md.) served as negative and positive controls, respectively. Synthesized cDNA of MDR1, MDR3, and β_2-microglobulin mRNA was amplified concomitantly. The PCR assays for each sample were carried out in duplicate.

Quantification of PCR Products

The PCR products were quantified by high-performance liquid chromatography (HPLC). The HPLC system consisted of the following: a high-precision pump (model 480 GT; Gynkotek, Germering, FRG), a degasser (Liliput; Gynkotek), a column oven (model STH 585; Gynkotek), a variable-wavelength UV monitor (model UVD-160; Gynkotek), and a biocompatible sample injection valve (model 9125; Rheodyne Inc., Cotati, Calif.) with a 20-μL sample loop. Separations of MDR1, MDR3, and β_2-microglobulin amplification products were performed on a stainless-steel column filled with nonporous reversed-phase particles using 0.1 *M* triethylammonium acetate (pH 7.0) as a mobile phase. Elution of these products was accomplished by a linear gradient of 10%-15% acetonitrile in 2 minutes. Column temperature was kept constant at 50 °C. The β_2-microglobulin mRNA expression is related to cell size. Since B-CLL is a rather uniform entity, only slight variations in the amount of β_2-microglobulin per cell can be assumed. Therefore, we used the HPLC-measured intensity of the β_2-microglobulin amplification product as the standard. The MDR1 and MDR3 expression levels of the 26 B-CLL samples were determined in percentages of β_2-microglobulin levels.

Statistical Analyses

The association between rhodamine 123 efflux and MDR1 and MDR3 mRNA expression and the clinical variables was analyzed by the Mann–Whitney *U* test and the chi-square test. The correlation between rhodamine 123 efflux and MDR1 and MDR3 data was estimated by simple regression analysis.

Results

Retention or Efflux of Rhodamine 123 in B-CLL

The rhodamine 123 efflux assay was applied to peripheral blood samples of all 42 B-CLL patients. In samples from 34 (81%) of the 42 patients, rhodamine

123 efflux was detectable. The percentage of cells showing rhodamine 123 efflux ranged from 10% to 70%, with a median of 31%. In the presence of multidrug resistance inhibitors, the efflux was completely blocked (Fig. 1). In eight (19%) of 42 patients, we found no rhodamine 123 efflux. In all rhodamine 123-positive B-CLL patients, the onset of rhodamine 123 efflux was rather quick and reached a steady state after approximately 2 hours. Statistical analysis revealed that the percentage of cells showing rhodamine 123 efflux was significantly lower among previously untreated patients than among previously treated patients ($P<.03$). However, rhodamine 123-negative

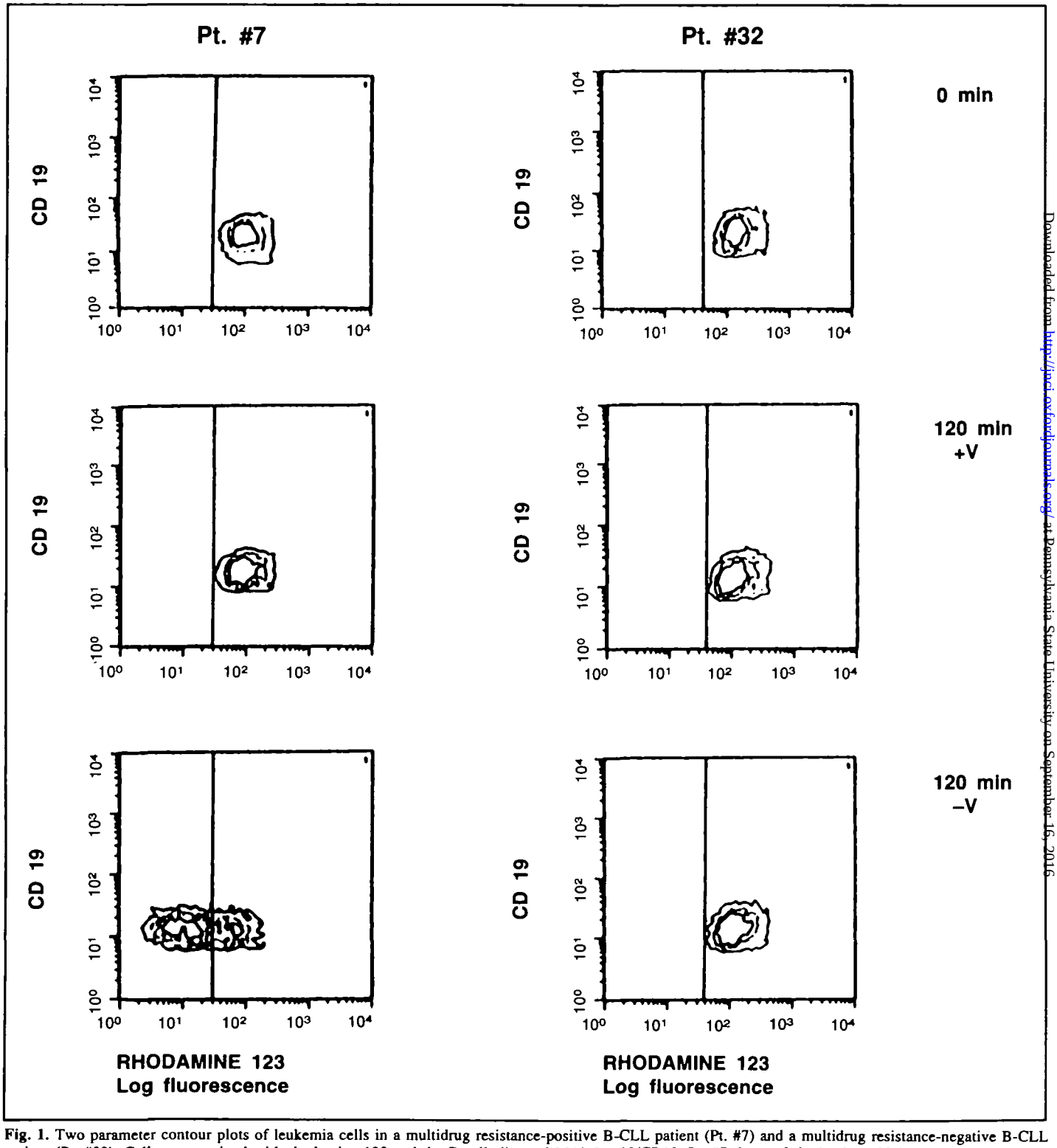

Fig. 1. Two parameter contour plots of leukemia cells in a multidrug resistance-positive B-CLL patient (Pt. #7) and a multidrug resistance-negative B-CLL patient (Pt. #32). Cells were stained with rhodamine 123 and the B-cell–directed MAb Leu12/CD19. Pt. #7: Loss of rhodamine 123 is clearly evident after 120 minutes in rhodamine 123-free medium (–V). The cells to the left of the cursor (58%) show rhodamine 123 efflux. Efflux is completely abolished in the presence of verapamil (+V). Pt. #32: No rhodamine 123 efflux is evident after 120 minutes.

cases were about equally distributed among untreated and previously treated patients (four of 20 and four of 22, respectively). Treatment regimens included at least one drug associated with multidrug resistance. The exception was the regimen of chlorambucil and prednisone received by one patient. High rhodamine 123 efflux activity in drug-exposed patients was not related either to one of the two treatment regimens (cyclophosphamide + vincristine + prednisone or mitoxantrone + prednimustine) or to a high cumulative dose of vincristine or mitoxantrone. There was no association between rhodamine 123 efflux and Rai stage, disease progression, absolute lymphocyte count, or duration of disease.

Analysis of blood samples, obtained from healthy volunteers (n = 5), showed rhodamine 123 efflux among the population of normal B lymphocytes. The percentage of normal B cells showing dye efflux ranged between 27% and 35%.

PCR Analysis

Peripheral blood samples from 26 patients were analyzed for the expression of MDR1 and MDR3 mRNA. MDR1, MDR3, and β_2-microglobulin mRNA levels were quantified by HPLC. Among the 26 patients analyzed, 25 showed detectable MDR1 mRNA levels, although these levels were consistently low and ranged between 1.8% (patient 18) and 25.8% (patient 17) of β_2-microglobulin mRNA. In addition, the patients' MDR1 mRNA levels were consistently below the MDR1 mRNA levels of the drug-resistant KB-8-5 cell line, which served as a positive control; the levels ranged between 2% and 16% of KB-8-5 cells, with a median of 7%. No statistically significant association between the levels of MDR1 mRNA and Rai stage, disease progression, or prior treatment was found.

MDR3 mRNA expression was found in all 26 patients analyzed. The expression levels ranged between 0.8% (patient 11) and 127.4% (patient 5) of β_2-microglobulin mRNA. There was a tendency toward higher MDR3 mRNA levels in Rai stages III or IV compared with stages 0-II, but it was not statistically significant ($P<.11$). No association was observed between MDR3 expression and prior treatment or disease progression.

Rhodamine 123 Efflux Versus PCR Analysis

As shown in Fig. 2, A, the levels of MDR1 mRNA expression correlated well with rhodamine 123 efflux ($r = .72$; $P<.0001$). Among the 26 patients analyzed for MDR1 mRNA expression, four rhodamine 123-negative patients were found. Three of these patients had low, but detectable, MDR1 mRNA levels (ranging from 3.6% to 6.2% of β_2-microglobulin mRNA), whereas one patient (patient 2)—in accordance with the efflux assay—was MDR1 mRNA negative. In contrast to MDR1 expression, there was no evidence of a correlation between MDR3 mRNA levels and rhodamine 123 efflux ($r = .23$; $P<.26$; Fig. 2, B). In particular, we found no rhodamine 123-positive and MDR3-positive patient who was MDR1 negative. Furthermore, there was no significant correlation between MDR1 and MDR3 mRNA levels ($r = .15$; $P<.46$; Fig. 2, C).

Serial Analysis of Rhodamine 123 Efflux

In order to determine the level of variation of rhodamine 123 efflux, we performed a second assay in 12 patients at intervals ranging from 7 to 10 months after the initial sample was taken (median, 8 months). All 12 patients had no change in Rai stage; four of them had received cytotoxic therapy between the two measurements. Two patients developed progressive disease as defined by an increase in lymphocyte counts and lymph node enlargement; 10 had stable disease. Two patients had a marked increase (>10%) in the percentage of cells showing rhodamine 123 efflux. One of these patients had received five cycles of chemotherapy with the combination of cyclophosphamide, vincristine, and prednisone between the two measurements, whereas the other patient remained untreated. In another patient, we observed a marked decrease (from 42% to 22%) in the cells showing rhodamine 123 efflux. This patient, who had not been exposed to cytotoxic treatment, developed progressive disease between the two assays. In the other nine patients, no change in the percentage of cells showing rhodamine 123 efflux was observed; in particular, the three rhodamine 123-negative patients remained negative. PCR analysis of MDR1 mRNA expression was performed in parallel and confirmed the results obtained by the efflux assay.

Discussion

Which multidrug resistance-screening technique is most suitable for clinical use? Much effort has been made to clarify this question, but as yet no single assay has been shown to meet both criteria of high sensitivity and of high specificity (*9,19,20*). Thus, there is general agreement that the exact evaluation of P-glycoprotein–expressing malignant cells in clinical samples requires the combination of more than one assay (*11,21*). In the present study, we combined a rhodamine 123 dye efflux assay of P-glycoprotein activity with a quantitative mRNA assay using reverse transcription and PCR to assess the level of MDR1/P-glycoprotein expression in patients with B-CLL. Recently, Chaudhary et al. (*22*) were able to demonstrate a direct relationship between P-glycoprotein activity and the efflux of the fluorescent dye rhodamine 123. In addition, this assay provides the possibility of dual fluorescence analysis and thus the possibility to measure selectively the rhodamine 123 efflux of phycoerythrin-labeled cell subsets. PCR analysis, on the other hand, offers the advantage of giving quantifiable results at low levels of MDR1 mRNA expression (*18,23*). Furthermore, both methods require only small amounts of tumor specimens. On the basis of these findings, we considered the combination of these two multidrug resistance-screening techniques to be suitable for clinical use.

The clinical role of MDR1 gene expression is less clear in chronic leukemias than in acute myeloid leukemia or multiple myeloma (*7,8*). For B-CLL, contradictory results have been published on the frequency of MDR1/P-glycoprotein expression (*6-8,24*). We

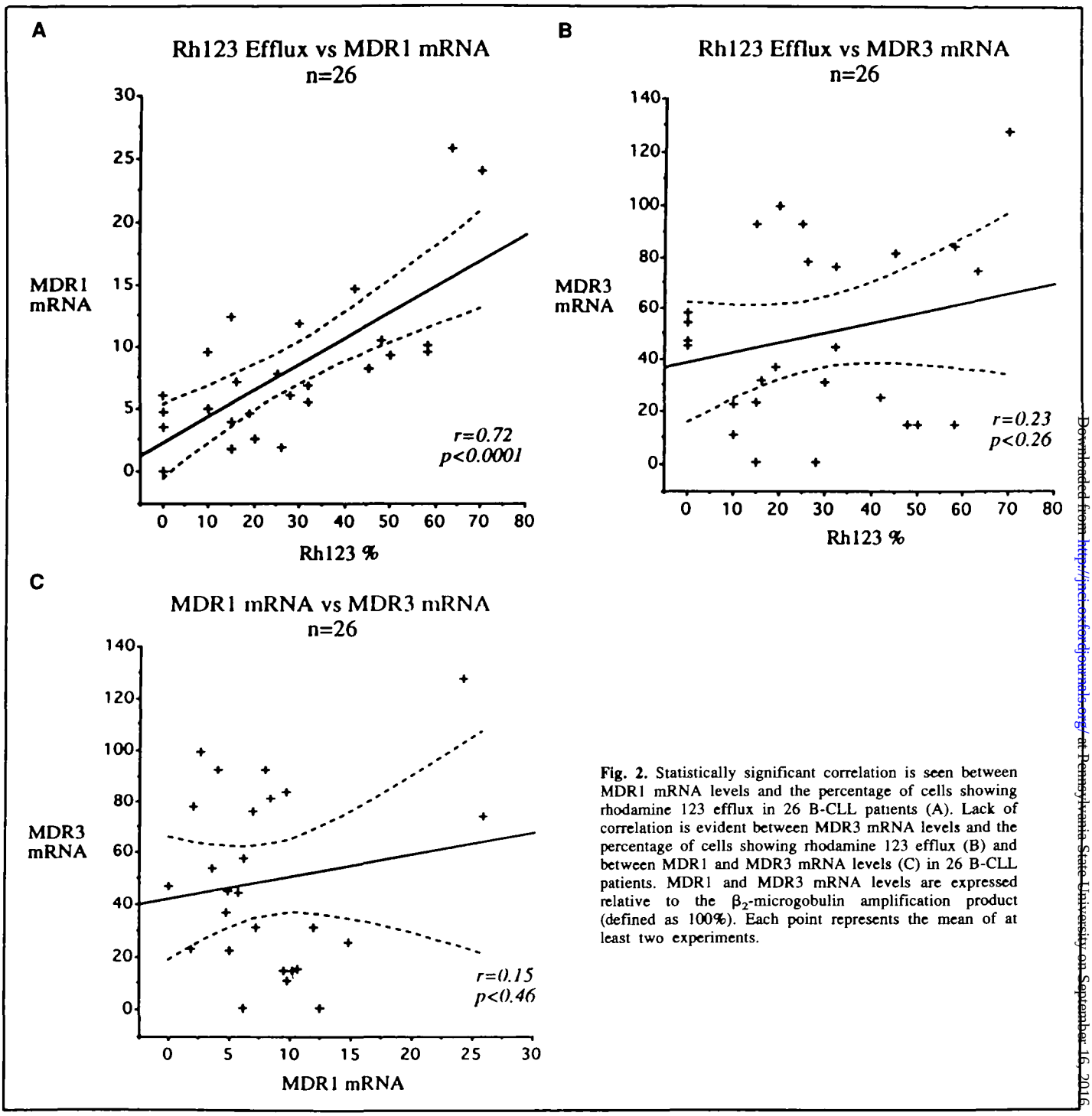

Fig. 2. Statistically significant correlation is seen between MDR1 mRNA levels and the percentage of cells showing rhodamine 123 efflux in 26 B-CLL patients (A). Lack of correlation is evident between MDR3 mRNA levels and the percentage of cells showing rhodamine 123 efflux (B) and between MDR1 and MDR3 mRNA levels (C) in 26 B-CLL patients. MDR1 and MDR3 mRNA levels are expressed relative to the β_2-microgobulin amplification product (defined as 100%). Each point represents the mean of at least two experiments.

found activity of P-glycoprotein, however, in 34 (81%) of the 42 B-CLL patients. The remaining eight rhodamine 123-negative patients were almost equally distributed among untreated (20%) and drug-treated (18%) patients. P-glycoprotein activity was evident in 10%-70% of the malignant B lymphocytes. The high incidence of rhodamine 123-positive cases in previously untreated patients provides evidence that P-glycoprotein expression in B-CLL patients is intrinsic rather than acquired. This hypothesis is further supported by the observation that peripheral B cells from healthy volunteers also show rhodamine 123 efflux. Thus, B-CLL could represent, at least in part, a hematopoietic malignancy arising from B lymphocytes that normally express the MDR1 gene.

In agreement with the results of other investigators (*7,24*), we could not detect a statistically significant association between P-glycoprotein expression and Rai stage, disease progression, or duration of disease. However, we found a lower percentage of cells showing rhodamine 123 efflux in untreated than in previously treated patients. This observation might reflect induction of enhanced P-glycoprotein activity and/or selection of the MDR1-expressing cells to subsequent courses of chemotherapy. For prognostic conclusions, survival analysis was not performed in our

study because of the relatively short follow-up. Nevertheless, we chose disease activity (progressive versus stable disease) as a variable that provides information concerning the aggressiveness of the disease. This information provided no evidence that increased MDR1/P-glycoprotein expression was associated with a more aggressive phenotype of tumor cells and thus contradicts a recent study (*23*) where an association was found in several solid tumors. In conducting serial analyses, we even found a decrease in P-glycoprotein activity in one patient who had developed progressive disease.

In our series of B-CLL patients, we initially tried to detect P-glycoprotein on the surface of the B cells by immunofluorescence staining with the MAb MRK16 (*25*), but we found this antibody to be of low sensitivity (data not shown). In contrast, we demonstrated a good correlation between the results of the rhodamine 123 efflux assay and the quantitative PCR-based MDR1 mRNA analysis; this correlation strongly indicates that the functional activity of P-glycoprotein was directly related to the level of MDR1 mRNA expression. To our knowledge, this correlation has not yet been demonstrated in clinical samples. Nevertheless, we found some discrepancy in three B-CLL patients without detectable rhodamine 123 efflux, who had low, but quantifiable, MDR1 mRNA signals. It has been suggested that the PCR technique provides the highest sensitivity for detection of MDR1 mRNA. However, since this assay requires homogenized material, it is possible that the observed MDR1 mRNA signals could be false-positive results caused by contaminating, MDR1-expressing nonmalignant cells (*26*). To minimize the risk of contamination, we processed only blood samples containing more than 60% B lymphocytes. However, it was only recently that P-glycoprotein activity was detected in several subsets of normal blood lymphocytes as well as in hematopoietic progenitor cells (*14,22*). Consequently, the accurate determination of even low levels of MDR1 expression in hematologic malignancies has to include the possibility of contamination by these nonmalignant cell subsets.

Another possible explanation for the rhodamine 123-negative, but MDR1-positive, patients might be that post-translational modification of P-glycoprotein altered its function without influencing the detection of MDR1 mRNA. There is strong evidence in the literature that phosphorylation of P-glycoprotein increases its pumping activity and that this reaction is mediated by protein kinase C (*12*). The detection of MDR1 expression at the mRNA or protein level, therefore, does not necessarily predict the actual level of the P-glycoprotein–pumping activity.

The role of MDR3 in clinical drug resistance is still unknown. MDR3 expression in human leukemias has been found to be restricted to cells of the B-cell lineage (*8*). Several investigators (*8,27*) suggested that the MDR3 gene encodes for a drug efflux pump and that overexpression of this gene might be related to drug resistance. With quantitative PCR, we were able to detect MDR3 mRNA in all B-CLL patients studied. Nevertheless, there was no evidence that overexpression of MDR3 was related to rhodamine 123 efflux. In addition, there was no correlation between MDR1 and MDR3 mRNA levels. In accordance with a recent study (*7*) that demonstrated high levels of MDR3 mRNA to be restricted to B-CLL patients with Rai stages III or IV, we also found a tendency toward higher MDR3 expression in patients with advanced stages; however, the number of patients with stage III or IV disease was rather small in our series. Whether or not MDR3 overexpression represents an independent prognostic parameter in B-CLL needs to be established.

In summary, our data demonstrate that the majority of B-CLL cases show MDR1 expression and P-glycoprotein activity. However, P-glycoprotein activity is not associated with the clinical course of the disease. Furthermore, our results proved the combination of an assay for protein activity with a PCR technique for quantifying mRNA to be most suitable for the detection of multidrug resistance-expressing cells in hematologic samples. Whether or not functional activity of P-glycoprotein, measured by rhodamine 123 efflux, is directly related to clinical drug resistance remains to be established in further studies.

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Notes

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Association of MMP-2 Activation Potential With Metastatic Progression in Human Breast Cancer Cell Lines Independent of MMP-2 Production

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Background: **Expression of matrix metalloproteinase-2 (MMP-2), the 72-kd type IV collagenase/gelatinase, by cancer cells has been implicated in metastasis through cancer cell invasion of basement membranes mediated by degradation of collagen IV. However, the abundance of this latent proenzyme in normal tissues and fluids suggests that MMP-2 proenzyme utilization is limited by its physiological activation rather than expression alone. We previously reported activation of this proenzyme by normal and malignant fibroblastoid cells cultured on collagen I (vitrogen) gels.** ***Purpose:*** **Our purposes in this study were 1) to determine whether MMP-2 activation is restricted to the more invasive human breast cancer cell lines and 2) to localize the activating mechanism.** ***Methods:*** **Zymography was used to monitor MMP-2 activation through detection of latent MMP-2 (72 kd) and mature species of smaller molecular weight (59 or 62 kd). Human breast cancer cell lines cultured on plastic, vitrogen, and other matrices were thus screened for MMP-2 activation. Collagen I-cultured cells were exposed to cycloheximide, a protein synthesis inhibitor, or to protease inhibitors to determine the nature of the MMP-2–activating mechanism. Triton X-114 (TX-114) detergent extracts from cells cultured on collagen I or plastic were incubated with latent MMP-2 and analyzed by zymography to localize the MMP-2 activator.** ***Results:*** **MMP-2 activation was only induced by collagen I culture in the more aggressive, highly invasive estrogen receptor-negative, vimentin-positive human breast cancer cell lines (Hs578T, MDA-MB-436, BT549, MDA-MB-231, MDA-MB-435, MCF-7_{ADR}) and was independent of MMP-2 production. MMP-2 activation was detected in cells cultured on collagen I gels but not in those cultured on gelatin gels, Matrigel, or thin layers of collagen I or IV, gelatin, or fibronectin. Collagen-induced activation was specific for the enzyme species MMP-2, since MMP-9, the 92-kd type IV collagenase/gelatinase, was not activatable under similar conditions. MMP-2 activation was inhibited by cycloheximide and was sensitive to a metalloproteinase inhibitor but not to aspartyl, serine, or cysteinyl protease inhibitors. MMP-2 activation was detected in the hydrophobic, plasma membrane-enriched, TX-114 extracts from invasive collagen I-cultured cells.** ***Conclusion:*** **Collagen I-induced MMP-2 activation is restricted to highly invasive estrogen receptor-negative, vimentin-positive human breast cancer cell lines, is independent of MMP-2 production, and is associated with metastatic potential. Our findings are consistent with plasma membrane localization of the activator.** ***Implications:*** **The MMP-2 activation mechanism may represent a new target for diagnosis, prognosis, and treatment of human breast cancer. [J Natl Cancer Inst 85:1758–1764, 1993]**

A variety of studies have implicated matrix metalloproteinase-2 (MMP-2), the 72-kd type IV collagenase/gelatinase, in cancer cell invasion of basement membranes and in subsequent metastasis (*1-3*). Penetration of basement membranes by cancer cells is believed to require the degradation of collagen IV, since this protein is a major structural component of the basement membrane (*4,5*). There are

*See "Notes" section following "References."