Clinical Significance of Multidrug Resistance P-Glycoprotein Expression on Acute Nonlymphoblastic Leukemia Cells at Diagnosis

By Lydia Campos, Denis Guyotat, Eric Archimbaud, Pascale Calmard-Oriol, Takashi Tsuruo, Jacques Troncy, Danielle Treille, and Denis Fiere

To evaluate the clinical value of the expression of multidrug resistance P-glycoprotein (P-170) on the surface of acute nonlymphoblastic leukemia (ANLL) cells, we analyzed specimens from 150 newly diagnosed patients for staining with MRK16, a monoclonal antibody (MoAb) that binds to an external epitope of P-170. Other surface markers (CD13, CD14, CD15, and CD34) were studied by the same technique. A marker was considered positive when 20% or more cells were stained. Of 150 samples, 71 were P-170-positive. These cases did not differ from P-170-negative cases with regard to age, sex, initial white blood cell (WBC) counts, or French-American-British (FAB) type (except for M3 ANLL, which was more frequently negative). However, leukemias arising from previous myelodysplastic syndrome (MDS) and therapy-induced leukemias were more frequently P-170-positive. CD34 and P-170 expression were significantly associated. All patients were treated by intensive chemotherapy. Complete remission (CR) rates were significantly lower in P-170-positive (23/71, 32%) than in P-170-negative cases (64/79, 81%) (P < 10^{-4}). CD34 positivity was also associated with a low remission rate (P < 10^{-4}). Survival was shorter for P-170- and CD34-positive patients (P < 10^{-3}). The prognostic value of both markers was confirmed in multivariate analysis. CR duration was also shorter for P-170-positive cases, but the difference is less significant (P = .05). It is concluded that P-170 analysis may be an important tool for predicting the outcome of intensive chemotherapy in ANLL patients.

DRUG RESISTANCE is the major cause of treatment failure in acute nonlymphoblastic leukemia (ANLL). The mechanisms by which leukemic cells are or become resistant to chemotherapy are still unclear. In vitro studies on tumor lines have shown that one mechanism implicates the multidrug resistance gene MDR-1, which encodes for a transmembrane glycoprotein (P-170) capable of expelling cytostatic drugs from the cytosol. An increase of MDR-1 RNA or of its product (P-170) has been reported in a large series of hematological malignancies, including multiple myeloma, non Hodgkin's lymphoma, chronic myelogenous leukemia in blast crisis, and acute lymphoblastic leukemia. In ANLL, this overexpression was frequently observed in patients in resistant or relapsed disease, but also in some patients at diagnosis or even in complete remission (CR). Despite this large number of studies, few prospective data are available regarding the clinical value of MDR-1/P-170 analysis in the blasts of ANLL patients at diagnosis. In a series of 36 patients, Sato et al showed a poor prognosis for those with high levels of MDR-1 RNA transcripts, while a preliminary report by Ball et al did not confirm the prognostic value of P-170 expression as detected by flow cytometry. In this study, we show that P-170 expression is associated with stem cell (CD34+) phenotype and with poor outcome of intensive antileukemic treatment.

MATERIALS AND METHODS

Patients. One hundred fifty patients with newly diagnosed ANLL presenting between June 1986 and December 1990 and treated by intensive chemotherapy were studied. Diagnosis was made on bone marrow smears according to usual cytological and cytochemical procedures, and classification was established according to French-American-British (FAB) criteria. Leukemias secondary to previous chemotherapy or to preceding myelodysplastic syndrome (MDS) were not excluded from the analysis. All patients gave informed consent to participate in the protocol.

Antileukemic treatment differed according to age and year of diagnosis, but for remission induction all patients received daunorubicin or mitoxantrone for 3 days and cytosine arabinoside for 7 days. CR was assessed according to Cancer and Leukemia Group B (CALGB) criteria. Details on induction and postinduction treatments are given in Table 1. Sixteen patients were treated by allogeneic bone marrow transplantation (nine in first remission and seven with relapsed or resistant disease), and were censored for analysis at the date of transplant.

Leukemic cells. Cells were aspirated from bone marrow in all cases, separated by Ficoll sedimentation (d = 1.077), washed, and resuspended in phosphate-buffered saline (PBS) or RPMI-1640. Cytospin samples showed that the percentage of blasts was always greater than 80% after separation. Cells were either analyzed immediately (N = 82) or cryopreserved with 20% fetal calf serum and 10% dimethyl sulfoxide (DMSO) and analyzed later after quick thawing (N = 68). Preliminary studies showed no difference in P-170 expression before and after cryopreservation (data not shown).

P-170 expression. P-170 expression was analyzed by indirect immunofluorescence, using the MRK16 monoclonal antibody (MoAb) as described by Tsuruo et al. Briefly, cells were fixed in 1% paraformaldehyde/PBS, washed twice, and incubated for 30 minutes at 4°C with 0.2 mL of MoAb solution. They were then washed twice in PBS, and fluorescein-conjugated F(ab') fragments (Bioart, Meudon, France) were added for 30 minutes at 4°C. Analysis was performed with a Facscan II cytometer (Becton Dickinson, Mountain View, CA). Negative controls were performed by incubating cells with normal mouse serum instead of MRK16 MoAb. Samples were considered positive when 20% more cells than the control were stained.

Surface marker analysis. Surface markers were analyzed with the following MoAbs: MY7 and My9 (Coulter Immunology, Hialeah, FL) directed, respectively, to CD13 and CD33 antigens;
Correlations with clinical and biological characteristics. There was no correlation between P-170 expression and initial characteristics such as sex, age, presence of extramedullary disease, blood counts, or marrow blast percentage. No relationship was noted to FAB subtype, with the exception of M3 subtype, where only two of 12 cases were P-170-positive ($P < .05$).

One hundred twenty-two ANLL cases were considered de novo, while 12 cases were secondary to preceding MDS and 16 to chemotherapy for previous malignancy. P-170 was more frequently expressed in ANLL secondary to MDS (9/12) and previous chemotherapy (10/16) than in de novo ANLL (52/122) ($P < .05$).

The relationships of P-170 to other surface markers were studied. CD13, CD14, CD15, and CD34 were positive in 20%, 71 of 150 samples studied (47%) were considered positive for P-170 expression.

### Table 1. Chemotherapy Protocols Used

<table>
<thead>
<tr>
<th>Course</th>
<th>LYLM protocol (75 patients, age $\leq 60$)</th>
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<tbody>
<tr>
<td>Course 1</td>
<td>Daunorubicin, 70 mg/m², d 1 to 3</td>
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<tr>
<td></td>
<td>Cytosine arabinoside, 200 mg/m², d 1 to 7</td>
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<tr>
<td></td>
<td>6-Thioguanine, 200 mg/m², d 1 to 7</td>
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<tr>
<td>Course 2</td>
<td>Cytosine-arabinoside, 3 g/m² x 2, d 1 to 4</td>
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<td>Amascrine, 90 mg/m², d 6 to 7</td>
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<tr>
<td>Course 3</td>
<td>Same as course 1, or Daunorubicin, 45 mg/m², d 1 to 3</td>
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<td>Cytosine arabinoside, 500 mg/m², d 1 to 3 and 8 to 10</td>
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<tr>
<td>LYLM 85 (32 patients, age $&gt; 60$)</td>
<td>Maintenance</td>
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<tr>
<td></td>
<td>Low-dose cytosine arabinoside for 12 mo</td>
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<tr>
<td>EORTC AML9 (44 patients, age $&gt; 60$)</td>
<td>Course 1</td>
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<tr>
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<td>Daunorubicin, 30 mg/m², d 1 to 3, or</td>
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<td></td>
<td>Mitoxantrone, 8 mg/m², d 1 to 3</td>
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<td></td>
<td>Cytosine arabinoside, 100 mg/m², d 1 to 7</td>
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<tr>
<td>Course 2</td>
<td>Daunorubicin, 30 mg/m², d 1, or</td>
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<td></td>
<td>Mitoxantrone, 8 mg/m², d 1</td>
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<td></td>
<td>Cytosine arabinoside, 100 mg/m², d 1 to 7</td>
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<td>Maintenance</td>
<td>Low-dose cytosine arabinoside or no maintenance</td>
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Cris-6 (a gift from Dr. Villegas, Barcelona, Spain) directed to CD14 antigen; SMy15a (Biosys, Compiègne, France) to CD15 antigen; My10 (Becton Dickinson) to CD34. Human AB serum was added to the cell suspension to avoid nonspecific fixation of the MoAb on Fc receptors. Cells were incubated with MoAbs at 4°C for 30 minutes, washed with PBS, incubated with F(ab')₂ fragments, and analyzed by indirect immunofluorescence. Controls were performed with a nonreactive antibody (MSlgM or MSlgG kits, Coulter Immunology). The staining was considered positive when 20% more cells than the control were stained.

**Statistical analysis.** The relationships of the expression of P-170 to quantitative parameters (age, blood counts, percent of marrow blasts) were studied by analysis of variance. The relationships to quantitative parameters (sex, FAB type, extramedullary disease remission) were analyzed by chi-square test (or Fisher’s exact test when either group included fewer than 20 cases).

Survival curves were plotted according to the method of Kaplan-Meier. Survival or remission durations of different groups were compared by the log-rank test. The respective influence of different parameters on survival or CR duration was calculated according to the Cox proportional hazards regression method. For this last analysis, the expression of P-170 and other surface markers was used as a continuous variable, and a logarithmic transformation of white blood cell (WBC) count was performed.

All calculations were performed using the BMDP statistical program (BMDP Statistical Software, Los Angeles, CA).

**RESULTS**

**P-170 expression.** The expression of P-170 was heterogeneous in terms of number of cells stained or fluorescence intensity. Figure 1 shows the number of samples according to the percentage of positive cells. With a cutoff point of 20%, 71 of 150 samples studied (47%) were considered positive for P-170 expression.
CD34 expression was also predictive of induction treatment outcome, as 28 of 83 (34%) CD34+ patients went into remission, versus 59 of 67 (88%) CD34− patients (P < 10−4). By combining both markers, it was possible to define a subgroup with a very poor prognosis (both markers positive, CR rate 9/50) and a subgroup with very good prognosis (both markers negative, CR rate 45/46).

Factors influencing survival were first studied by univariate analysis. Survival was significantly shorter for CD34+ (P < 10−5) and P-170−positive patients (P < 10−5) (Fig 2). Age 60 or less was also significant (P = .01). In multivariate analysis, the significant variables were P-170 (P < 10−5) and CD34 expression (P < .005), and age (P < .01). The presence of preceding myelodysplastic syndrome or secondary leukemia did not significantly influence survival, nor did initial counts, FAB classification, or other surface markers.

The remission duration was studied in 87 patients by univariate analysis, there was no significant factor, but patients with de novo disease, and with P-170−negative disease showed a trend toward longer remission (.05 < P < .1). In multivariate analysis, P-170 expression significantly influenced CR duration (P = .05).

**DISCUSSION**

The expression of MDR-1 products has already been reported in a large number of ANLL patients, mainly in refractory or relapsed disease, but also at diagnosis.10 The percentage of untreated cases expressing MDR1 obviously depends on the method used for detection. In our study using MRK16 MoAb and the staining technique described by Hamada and Tsuuro,14 we observed 47% positive cases. This is consistent with the results of Sato et al, who reported a high expression of MDR-1 RNA transcripts in 36 untreated patients. This percentage is much higher than that reported by Kennitz et al.12 on a larger series of patients using a polyclonal antibody and an immunocytochemical technique. Like Sato et al, we did not observe a correlation with initial characteristics such as age, hematological parameters, or FAB classification (with the exception of FAB M3), and we confirmed the higher expression in therapy-related leukemias. This last finding might be explained by previous exposure to chemotherapy, but we also observed a high expression in ANLL secondary to MDS, which confirms the observation of Holmes et al.11 It is possible that P-170 is expressed more in leukemias arising from poorly differentiated cells. The partial correlation with CD34 is consistent with this hypothesis, and was recently reported by List et al in a series of 45 cases with MDS and therapy-induced ANLL.19

High levels of MDR-1/P-170 in malignant cells have been associated with clinical resistance to chemotherapy in a variety of malignancies, including ovarian cancer, neuroblastoma, myeloma, and acute leukemia. However, few prospective studies have been conducted in patients with newly diagnosed ANLL. In the series of Sato et al, a higher CR rate and a longer remission duration were observed in patients with low levels of MDR-1 transcripts. We also observed a significant difference between P-170−positive and −negative patients with regard to CR achievement and survival. The difference was less significant for CR duration, but this may be explained by the fact that there were too few patients in the P-170−positive group in remission, and by the heterogeneity of postinduction treatments. Our results are in contradiction with those of Ball et al, although we used the same MoAb to detect P-170. Detailed analysis of this last study is needed to understand this discrepancy. Our study also confirmed the prognostic value of CD34 that we and others have already reported.

Although our data indicate that P-170 expression is highly correlated with therapy outcome, MDR is certainly not the only factor of drug resistance in ANLL. However, in vitro data show that drug resistance of ANLL cells to daunorubicin may be reversed by P-170 blockers such as verapamil. Our study contributes to the identification of patients with poor prognosis, but also suggests that therapeutic trials using P-170 blockers in addition to chemotherapy might be of interest, at least for patients with MDR-1−positive blast cells.

**REFERENCES**

Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis [see comments]

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