Predictive Value for Treatment Outcome in Acute Myeloid Leukemia of Cellular Daunorubicin Accumulation and P-Glycoprotein Expression Simultaneously Determined by Flow Cytometry

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To evaluate the clinical relevance of multidrug resistance (MDR) phenotype, the intracellular daunorubicin accumulation (IDA) and P-glycoprotein (P-gp) expression were investigated in 87 adult patients with acute leukemia: 99 patients with de novo acute myeloid leukemia (AML), 10 with AML at relapse, and eight with secondary leukemia to myelodysplastic syndromes (MDS-AML). IDA and P-gp expression were determined by double-labeling flow cytometry analysis. Of 87 patients, 36 expressed P-gp (41%). P-gp expression was more frequently observed in AML at relapse and MDS-AML as compared with de novo AML (P = .0001). P-gp expression was significantly associated with CD34 expression (P = .0003) and chromosome 7 abnormalities (P = .027). A significantly reduced IDA was observed in P-gp+ as compared with P-gp- patients (P = .0007). Of the 87 patients, 51 achieved complete remission (CR). A reduced IDA was observed in patients in failure as compared with patients in CR (22% ± 17% v 42% ± 21%; P = 10-4). Twelve of 36 P-gp+ patients as compared with 40 of 51 P-gp- patients achieved CR (33% v 78%; P = 10-4). The prognostic value of IDA and P-gp expression was confirmed in multivariate analysis. These data suggest that the determination of IDA and P-gp expression may be useful in designing therapy for patients with AML.

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D RUG RESISTANCE is believed to be the major cause of failure of chemotherapy in acute myeloid leukemia (AML). Multidrug resistance (MDR) is a phenotype of resistance to several structurally unrelated cytotoxic agents, such as anthracyclines, vinca alkaloids, and epipodophyllotoxins.1 MDR is associated with the overproduction of a 170-kD membrane glycoprotein (P-gp) that is encoded by the MDR1 gene located on the long arm of chromosome 7. P-gp functions as a transmembrane drug efflux pump, expelling the drug from the cell.2 An association between MDR1 RNA expression in leukemic cells and response to chemotherapy in AML has been suggested.3-5 A negative relationship between treatment outcome and P-gp expression, determined with the monoclonal antibody C219 or MRK16, has also been demonstrated.6-8 The clinical outcome of chemotherapy treatment was also studied by the drug accumulation into leukemic cells. Anthracyclines have been widely studied, as their fluorescent properties facilitate quantification of the intracellular fluorescence (ie, anthracycline drug content) by flow cytometry.9 However, the association between in vitro anthracycline accumulation and treatment outcome is not yet established.10-12 Our preliminary results demonstrated that intracellular daunorubicin fluorescence was highly correlated with clinical response of adult AML to chemotherapeutic regimens containing MDR-related drugs.13

The present study was designed to evaluate the clinical relevance of MDR phenotype in AML. We investigated the intracellular daunorubicin accumulation (IDA) and the relationship of this parameter with P-gp expression, hematologic features, and treatment outcome. IDA and P-gp expression were determined by double-labeling flow cytometry analysis in bone marrow samples from 87 patients with AML.

MATERIALS AND METHODS

Patients. The study included 87 adult patients with AML (46 males, 41 females) who were considered eligible for the response to chemotherapy. Mean age was 51.2 years (range, 16 to 79 years). Preliminary results on a smaller series of patients (23 of 87) have been previously reported.13 Sixty-nine patients presented with de novo AML, 10 with AML in relapse after a conventional treatment (anthracycline, cytosine-arabinoside), and eight with a blastic transformation of myelodysplastic syndromes (MDS-AML). Four patients were studied both at diagnosis and at relapse. For induction treatment, all patients with de novo AML received 45 mg/m2 of daunorubicin (n = 8), rubidazone (n = 17), or idarubicin (n = 45) according to age for 3 days and 200 mg/m2 of cytosine-arabinoside for 7 days. Patients at relapse and with AML secondary to MDS were treated with 12 mg/m2 x 3 days of mitoxantrone alone (n = 7) or combined with quinine, 30 mg/kg/d for 6 days (n = 10), or 45 mg/m2 x 3 of idarubicin (n = 1) and cytosine-arabinoside (1 g/m2 x 12). Complete remission (CR) was defined as the presence of less than 5% blast cells in a cellular bone marrow (BM) smear, the absence of circulating blasts and extramedullary leukemic infiltration, and a return to normal peripheral blood cell count within 4 weeks after the beginning of the chemotherapy. Patients who did not achieve a CR were classified according to the proposals of Preiser.14 Patients with a partial response were grouped with those patients that had no response and were termed nonresponders (NRs).

BM collection and preparation. Each BM sample was obtained during routine cytology sample collection. BM samples were layered onto Histopaque (density, 1.077; Sigma, St. Quentin Fallavier, France) and centrifuged at 1,500g for 30 minutes. The leukocyte fraction was collected by aspiration and resuspended in phenol red-free RPMI 1640 medium. When the original BM samples contained less than 50% atypical blast cell, the fraction obtained after separation was cytologically recontrolled using the May-Grumwald-Giemsa staining procedure and characterized before being analyzed. Among 87 samples, 23 contained more than 85% atypical blast cells; 49 contained 50% to 85% atypical blast cells (mean ± SD, 75% ± 7%).

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and were found to be enriched after Histopaque (92% ± 3%). The 15 other samples, which included six samples from the leukemic-transformed MDS, contained less than 50% atypical blast cells (38% ± 4%) and were enriched up to 75%.

Flow cytometry. Flow cytometric analyses were performed with an Orthocyte flow cytometer (Ortho Diagnostic Systems, Roissy, France). Double-labeling flow cytometry analyses were performed according to a method described previously. 12,13 IDA was assayed by incubating the cells with 2 mmol/L daunorubicin (Cerubidine; Laboratoire Roger Bellon, Neuilly, France) for 1 hour at 37°C. P-gp expression was analyzed by indirect immunofluorescence using the MRK16 monoclonal antibody (MoAb) at a final concentration of 10 μg/mL for 1 hour at 4°C coupled to fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG serum (Dako, Versailles, France) for 30 minutes at 4°C. Dead or membrane-altered cells were identified by addition of 75 μmol/L propidium iodide.

The atypical cell population was selected from double light-scattering analysis (forward angle and right angle light scattering). The two red fluorescence signals of daunorubicin and propidium iodide were analyzed using a logarithmic amplification. Each analysis was performed on at least 5,000 cells. IDA was determined by mean cellular fluorescence (mean ± SD) provided by the median channel number on the single-parameter red fluorescence histogram for each sample.

CD34 surface marker was analyzed by indirect immunofluorescence with My10 (Becton Dickinson, Mountain View, CA) MoAb directed against CD34 antigen. A nonrelevant mouse IgG was used as negative control for background fluorescence. Staining was considered positive when more than 15% of the cells were stained.

Statistical analysis. All analyses were performed using the BMDP statistical program (BMDP Statistical Software, Los Angeles, CA). The relationships of the expression of P-gp and IDA to qualitative parameters were studied by the χ^2 test or the Fisher’s exact test. The influence of different parameters on treatment outcome was calculated according to the Cox proportional hazard regression method. For this analysis, IDA and P-gp and CD34 expression were used as continuous variables.

RESULTS

IDA. Large variations were observed in leukemic samples: the mean proportion of IDA was 33.9 ± 21.9% (range, 1.2 to 92%). There was no significant difference between de novo AML (35.9 ± 22.1 SD) and AML at relapse and MDS-AML (26.7 ± 19.8 SD; P = .11). A statistically significant difference (P = .0007) in daunorubicin fluorescence was found between P-gp-positive and –negative samples with a threshold for positivity set to 10%, with respective means of 24.7 ± 17.1 (n = 36) and 40.5 ± 22.6 (n = 51). Figure 1 shows the percentage of samples according to the amount of IDA in P-gp-positive (Fig 1A) and –negative (Fig 1B) samples. A significant inverse relationship was observed between IDA and P-gp expression when they were used as continuous variables (r = −.348; P = .001).

Expression of P-gp. The expression of P-gp was heterogeneous in terms of number of cells stained. The mean proportion of MRK16-FITC–labeled cells was 14.27% ± 17% (range, 0.03% to 66%). Figure 2 shows the number of samples according to the percentage of positive cells. The mean proportion was significantly higher in AML at relapse and in MDS-AML (25.44% ± 16.32%) as compared with de novo AML (11.39% ± 16.09%; P < .0015). With a 10% positive cell cut-off, P-gp expression was detected in 36 of 87 (41.4%) BM samples, more frequently in AML in relapse and secondary to MDS (15 of 18) than in de novo AML (2 of 69; P = .0001).

Correlations with hematologic characteristics. No association was observed between age of patients, white blood cell count, absolute blast cell count, or French-American-British (FAB) subtype and IDA and P-gp expression. The expression of CD34 was available in 81 samples. CD34 was positive in 11 samples (cut-off point at 15%), and nine of them were P-gp–positive (cut-off point at 10%), while 22 of 70 CD34-negative samples were P-gp–positive. This association was statistically significant (P = .0014). When using the percentages of stained cells as continuous variables, a significant correlation was found (r = .39; P = .0003). Cytogenetic analysis was available on 68 samples. Twenty-four samples exhibited a normal karyotype, and seven of these (29.2%) expressed P-gp, while 20 of 44 samples (45.5%) with abnormal karyotype expressed P-gp (P = .21). Of the 44 samples with abnormal karyotype, 10 had monosomy 7 or chromosome 7 long arm deletion, and eight expressed P-gp. This association was statistically significant (P = .027; Table 1). Two of six MDS-AML patients with available karyotype exhibited chromosome 7 abnormality, while the eight others were observed in de novo AML. No significant relationship was observed between CD34 expression and chromosomal abnormalities.

Correlations with treatment outcome. CR was obtained.
P-gp expression (less than 10% positivity) showed a trend towards longer remission \( (P = .078, \text{data not shown}) \).

**DISCUSSION**

Our prospective study demonstrated that IDA and P-gp expression in the leukemic cells are independent prognostic factors with regard to treatment outcome in AML and suggested that MDR has a clinical importance in AML. Low IDA and elevated P-gp expression were associated with a lower CR rate. Moreover, increased drug resistance and expression of the MDR phenotype, designated as P-gp expression, have been observed in MDS-AML and in AML at relapse.

The clinical relevance of MDR phenotype in human hematologic malignancies is not yet established. In AML, most chemotherapeutic protocols involve MDR-related drugs at diagnosis and at relapse. Moreover, drug resistance remains a major problem in AML and explains the poor prognosis observed in certain cases. MDR studies in AML have been performed by many investigators, but results are still difficult to compare as the techniques used to detect MDR phenotype are different. They are mainly based on the detection of MDR1 RNA or P-gp expression in immunoassays or in flow cytometry analyses and assessment of anthracycline uptake. In the present study, we used a double-labeling flow cytometry analysis, which allowed the simultaneous determination of the expression of P-gp as a structural parameter and the accumulation of daunorubicin as a functional parameter. This procedure was optimized in experiments with the K562 human leukemic cell line and the P-gp-expressing K562-DXR, selected for its resistance to doxorubicin, as previously published.\(^5\)

In this study, we detected P-gp expression in 41.4% of leukemic samples. These data are consistent with those previously reported by investigators using the same technique, i.e., indirect immunofluorescence assay with MRK16 antibody.\(^7,20\) However, in another study, P-gp was not detected in any sample from patients with AML who were studied at

### Table 1. Distribution of Karyotype in Relation to AML Blast Cell Phenotype (P-gp and CD34 Expression) and Treatment Outcome

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Total ( n = 68 )</th>
<th>P-gp(^+)</th>
<th>CD34(^+)</th>
<th>CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>24</td>
<td>7</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>t(15;17)(q22;q12-21)</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>t(3;21)(q26;q21)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>inv(16)(p13;q22)</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Trisomy 8</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Trisomy 9</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>t(8;21)(q22;q22)</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Monosomy 7 or del(7)(q22-q36)</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Complex with monosomy 7 or del(7)(q22-q36)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Complex without chromosome 7 abnormality</td>
<td>15</td>
<td>5</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>

P-gp\(^+\) samples defined as more than 10% blast cells staining with MRK16 MoAb. CD34\(^+\) samples defined as more than 15% blast cells staining with My10 MoAb.
diagnosis and at relapse. \(^{21}\) We confirmed that P-gp was more frequently detected in AML evolving from MDS and in AML at relapse than in de novo AML (100% and 70.5% v 30%, respectively). \(^{2,20,22}\) As the patients with MDS-AML had not received any chemotherapy before our investigation, this difference cannot be explained by a therapy-induced change in P-gp expression. This may be due to the onset of the disease from poorly differentiated cells. As reported by other investigators, \(^{6,23,24}\) the expression of P-gp in MDS is associated with a stem cell phenotype. In our study, we confirmed these findings, as CD34-positive samples were found in five of eight MDS-AML patients, and a correlation between CD34 and MRK16 expression was observed. At relapse, the high percentage of leukemic cells expressing P-gp may be explained by previous exposure to chemotherapy, especially to MDR-related drugs such as anthracyclines. This finding was confirmed in patients who could be sequentially studied. Two of four showed an increase in MRK16-labeled cells (25% and 54.5% at relapse v 1.1% and 0% at diagnosis, respectively), and two were unchanged. Three patients are relapsing, two of them showed an increase of P-gp-positive cells, and one was unchanged. These data and the findings in 10 patients at relapse confirmed the progressive emergence of drug-resistant clones as described by other investigators. \(^{6,20,25}\)

The \textit{MDRI} gene is located on the long arm of chromosome 7 in chromosomal region \(7 q 21.22.\) \(^{26,27}\) An abnormal karyotype involving chromosome 7 is frequently found in patients with secondary leukemia and MDS-AML and is related to a poor response to chemotherapy. \(^{28}\) In our study, a significant association between chromosome 7 abnormality and P-gp expression was noted, and the CR rate for AML with chromosome 7 abnormality (20%) was significantly lower when compared with that for AML with normal karyotype (75%) or with another cytogenetic abnormality (70.5%). This might be due to P-gp expression, because this was more frequently expressed in AML with chromosome 7 abnormality (80%) as compared with AML with a normal karyotype (29.2%) or with another cytogenetic abnormality (35.3%). These data are consistent with those reported by other investigators, suggesting that immature cells express CD34 and the MDR phenotype. \(^{22,29}\)

We observed a good correlation between IDA and the clinical response. However, discordant reports have been published concerning the correlation between in vitro IDA and response to chemotherapy. Marie et al. \(^{17}\) observed a good correlation between the proportion of daunorubicin (DNR)-positive cells and the clinical response to a regimen including one MDR-dependent drug. \(^{13}\) Recently, Galetti et al. \(^{10}\) showed that a significant difference in the cellular DNR and its metabolite daunorubicinol was observed between responders and nonresponders with AML. On the other hand, Kessel et al. \(^{11}\) Kokenberg et al. \(^{16}\) and Campos et al. \(^{12}\) did not demonstrate a correlation between drug responsiveness and IDA. Maruyama et al. \(^{31}\) showed that the effects of verapamil on IDA were significantly greater in nonresponders than in responding patients with AML, but they did not determine the IDA in each group of patients.

The clinical significance of MDR phenotype has been assessed in recent studies. As reported by Sato et al. \(^{3} \) Pirker et al. \(^{4}\) and Marie et al. \(^{5}\) elevated \textit{MDRI} RNA levels were associated with lower CR rate. However, no association between \textit{MDRI} expression and CR achievement was found by

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### Table 2. Treatment Outcome of Patients by IDA and AML Blast Cell Phenotype P-gp and CD34 Expression

<table>
<thead>
<tr>
<th>Characteristic Step</th>
<th>Log Likelihood</th>
<th>Relative Risk (95% CI)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDA</td>
<td>1</td>
<td>42.3</td>
<td>1.06 (1.02-1.10)</td>
</tr>
<tr>
<td>Disease status*</td>
<td>2</td>
<td>37.7</td>
<td>0.10 (0.015-0.65)</td>
</tr>
<tr>
<td>P-gp expression</td>
<td>3</td>
<td>35.5</td>
<td>0.96 (0.93-1)</td>
</tr>
</tbody>
</table>

CD24 determination was available in 81 of 87 patients. IDA and P-gp expression used as continuous variables.

Abbreviation: CI, confidence interval.

*De novo AML \((n=69)\) v MDS-AML \((n=8)\) and AML at relapse \((n=4)\).

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Fig 3. Distribution of the mean proportion of IDA according to the clinical response in 87 patients with AML.
Gruber et al.32 Expression of P-gp in leukemic cells was also shown to be an independent prognostic factor, being associated with a lower CR rate.44 In the series reported by Sato et al.,2 a longer remission duration was observed in patients with low levels of MDR1 transcripts. In our study, the difference was not significant and may be explained by the small number of P-gp-positive patients in remission.

There is discrepancy in the above-cited studies concerning the relationship of IDA and/or MDR expression with response to treatment and their significance as prognostic indicators. This may be explained by the variability of the in vitro methodology (ie, MDR1 expression or immunohistochemical/flow cytometric detection of P-gp with the MRK16 or C219 MoAbs) and by the difficulty in defining the cutoff for positivity. For example, with MRK16, 20% was the cutoff used by Campos et al.7 and 10% was used by Solary et al.20 In our study, similar results were observed using a 10% or 20% cutoff value for MRK16 expression (data not shown). In addition, nearly all patients with AML are treated with DNR or a new anthracycline analogue (idarubicin) or anthra-cenedione (mitoxantrone) and cytosine-arabinoside (Ara-C). The antileukemic effect of Ara-C may explain the confounding results concerning the relationship of MDR phenotype with clinical response. Recently, Lacombe et al.13 studied Ara-C resistance using bromodeoxyuridine/DNA staining in patients with AML. They showed that the Ara-C resistance test was the best predictive test of remission outcome and was not correlated to P-gp activity. Further studies in which MDR phenotype and Ara-C resistance are determined may provide more insight into the role of MDR in clinical resistance in AML.

As did Loos et al.,34 we observed an inverse correlation between IDA and P-gp expression. Moreover, we found that a significant decrease in IDA was observed in P-gp-positive samples (P = .0007). This confirmed our data15 and the data published by Nooter et al.,35 who demonstrated that MDR1 gene overexpression in chemotherapy-resistant AML cells was associated with a decreased IDA that could be restored by cyclosporin-A. Enhancement of IDA by verapamil was noted by other investigators.31,36 In our previous study, we found functional evidence of enhancement of DNR by different modulators. This was confirmed in this study, as a significant increment of DNR by at least one reversing agent was observed in 75% of patients (data not shown). However, the relationship of the functional MDR phenotype with clinical response reported by Maruyama et al.31 was not confirmed by Ross et al.37 Moreover, as reported by Campos et al.12 and Marie et al.13, a subgroup of our patients had no IDA and no P-gp expression (n = 18). So, the expression of P-gp might not be the sole explanation for the low IDA. This situation could be explained by the presence of non-P-gp mechanisms of resistance as described in cell lines. Recently, HL60 cells (HL60/ADR) selected for anthracycline resistance have been isolated and characterized.38,39 These cells are defective in the cellular accumulation of drug but do not overexpress MDR1 gene and do not contain detectable levels of P-gp.38,40 Further studies suggested that resistance in HL60/ADR cells was related to the intracellular distribution of DNR in resistant cell lines, with a redistribution of DNR from the nucleus into cytoplasmic vesicles.41-43 Recently, a novel resistance-associated gene was found to be overexpressed in a non-P-gp MDR small-cell lung cancer cell line.44 This gene, called multidrug resistance-associated protein (MRP), encodes a membrane protein of 190 kD (P-190) that may be responsible for the decreased cellular drug accumulation observed in non-P-gp MDR cell lines such as the HL60/ADR cell line.45 Preliminary results in patients with acute leukemia showed that high expression of MRP was occasionally observed in AML.46 Further clinical studies are needed to characterize this non-P-gp MDR phenotype and to determine the potential effect of the well-known MDR reversal agents on non-P-gp MDR cells.

The prognostic value of MDR parameters, determined by P-gp or MDR1 gene expression and anthracycline accumulation, has been primarily analyzed separately in published reports. Despite sometimes discordant results, it seems that MDR phenotype detected by different methods may influence treatment outcome in patients with AML. In the study presented here, we were able to show that IDA and P-gp expression were of prognostic value in predicting the clinical response in patients with de novo AML, AML at relapse, and MDS-AML treated with chemotherapy regimens containing MDR-related drugs. The presence of functional resistance as defined by reduced IDA was significantly associated with a lower CR rate in AML. However, insights into non-P-gp mechanisms of resistance and effects of MDR modulators could provide a better understanding of chemotherapy resistance in AML and lead to a better therapeutic approach to improve the prognosis of AML.

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Predictive value for treatment outcome in acute myeloid leukemia of cellular daunorubicin accumulation and P-glycoprotein expression simultaneously determined by flow cytometry

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