High Expression of bcl-2 Protein in Acute Myeloid Leukemia Cells Is Associated With Poor Response to Chemotherapy

By Lydia Campos, Jean-Pierre Rouault, Odile Sabido, Pascale Oriol, Nora Roubi, Christian Vasselon, Eric Archimbaud, Jean-Pierre Magaud, and Denis Guyotat

The BCL-2 proto-oncogene encodes a mitochondrial protein that blocks programmed cell death. High amounts of bcl-2 protein are found not only in lymphoid malignancies, but also in normal tissues characterized by apoptotic cell death, including bone marrow. Using a monoclonal antibody to bcl-2 protein, we analyzed 82 samples of newly diagnosed acute myeloid leukemia. The number of bcl-2+ cells in each sample was heterogeneous (range, 0% to 95%), with a mean of 23%. The percentage of bcl-2+ cells was higher in M4 and M5 types, according to French-American-British classification, and in cases with high white blood cell counts. bcl-2 expression was also correlated with that of the stem cell marker CD34. In vitro survival of leukemic cells maintained in liquid culture in the absence of growth factors was significantly longer in cases with a high percentage of bcl-2+ cells. High expression of bcl-2 was associated with a low complete remission rate after intensive chemotherapy (29% in cases with 20% or more positive cells vs 85% in cases with less than 20% positive cells, P < 10−4) and with a significantly shorter survival. In multivariate analysis, the percentage of bcl-2+ cells (or the blast survival in culture), age, and the percentage of CD34+ cells were independently associated with poor survival.

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**MATERIALS AND METHODS**

**Patients.** This study included 82 consecutive patients with newly diagnosed AML who were treated by intensive chemotherapy. Diagnosis was performed on BM smears according to usual cytologic and cytochemical procedures and classification established according to the French-American-British (FAB) criteria. Chemotherapy varied according to age and year of treatment, but all patients received for remission induction an anthracycline drug or mitoxantrone for 3 to 5 days and cytosine-arabinoside (araC) for 7 days. Complete remission (CR) was assessed according to the Cancer and Leukemia Group B criteria. Postinduction treatment consisted of two intensive courses for patients 60 years of age or less and of consolidation with daunorubicin and araC and/or low-dose araC for patients older than 60 years of age. Nine patients were treated by autologous BM transplantation (6 in first remission and 3 with relapsed or resistant disease) and were censored for analysis at the date of transplantation. Details on antileukemic treatments are given in Table 1.

**Leukemic cells.** Cells were aspirated from BM in all cases, separated by Ficoll sedimentation (d = 1.077), washed, and resuspended in phosphate-buffered saline (PBS) or RPMI 1640. Cytocentrifuge samples showed that the percentage of blasts was always higher than 80% after separation. Cells were either used immediately or cryopreserved with 20% fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO) for later analysis after quick thawing. For immunocytochemical procedure, cells were cytocentrifuged and dried slides were stored at −20°C.

In addition to patient cells, the following cell lines were studied: KG1a (myeloblastic), HL60 (promyelocytic), Daudi (Burkitt line), and Val [lymphoma line with t(14;18) translocation].

**bcl-2 expression.** bcl-2 expression was analyzed using the anti-bcl-2 monoclonal antibody (MoAb) bcl-2/124 by indirect immunofluorescence (IF) and by immunoalkaline phosphatase (APAAP) techniques. The study was performed either with fresh cells or with cryopreserved cells, after confirming that cryopreservation and thawing did not modify the results (data not shown). For IF, 10^6 cells per sample were permeabilized for 30 minutes in 1 mL of PBS with 70% ethanol, washed twice, and resuspended for 10 minutes at 4°C in PBS with 1% human AB serum. Cells were then incubated for 30 minutes at 4°C with MoAb at optimal concentration (1:50 dilution of the culture supernatant). After two washings in PBS, fluorescein-conjugated goat antimouse F(ab') fragments (Biocon, Meudon, France) were added for 30 minutes at 4°C. Negative controls were performed by incubating cells with normal mouse serum instead of anti-bcl-2 MoAb. Fluorescence analysis was immediately performed with a

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Table 1. Chemotherapy Protocols Used

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Patients aged</th>
<th>Course 1</th>
<th>Course 2</th>
<th>Course 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYLAM</td>
<td>60 yrs or less</td>
<td>Daunorubicin 70 mg/m², days 1 to 3</td>
<td>AraC 200 mg/m², days 1 to 7</td>
<td>Same as course 1, or Daunorubicin 45 mg/m², days 1 to 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AraC 200 mg/m², days 1 to 7</td>
<td>AraC 200 mg/m², days 1 to 7</td>
<td>AraC 500 mg/m², days 1 to 3 and 8 to 10</td>
</tr>
<tr>
<td>GOELAM</td>
<td>60 yrs or less</td>
<td>Idenubicin 8 mg/m², days 1 to 5</td>
<td>AraC 200 mg/m², days 1 to 4</td>
<td>Etoposide 100 mg/m², days 1 to 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rubidazone 200 mg/m², days 1 to 7</td>
<td>AraC 200 mg/m², days 1 to 7</td>
<td>Amsacrine 100 mg/m², days 1 to 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AraC 3 g/m² × 2, days 1 to 4</td>
<td>Rubidazone 8 mg/m², days 5 to 6</td>
<td>EORTC AML9 (39 patients aged more than 60 yrs)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Idanubicin 3 g/m² × 2, days 1 to 4</td>
<td>Idanubicin 8 mg/m², days 5 to 6</td>
<td>Course 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AraC 200 mg/m², days 1 to 7</td>
<td>Rubidazone 200 mg/m², days 5 to 6</td>
<td>Amsacrine 100 mg/m², days 1 to 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AraC 8 mg/m², days 1 to 4</td>
<td></td>
<td>AraC 100 mg/m², days 1 to 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Same as course 1, or Daunorubicin 45 mg/m², days 1 to 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AraC 500 mg/m², days 1 to 3 and 8 to 10</td>
<td></td>
<td>Maintenance Low-dose AraC or no maintenance</td>
</tr>
</tbody>
</table>

Faedar Plus cytometer (Becton Dickinson, Mountain View, CA) equipped with an 8 W argon ion laser (Innova 90, Coherent, Palo Alto, CA) emitting 250 mW at 488 nm. Green fluorescence signals were collected through a bandpass filter (530/30 nm). All signals were processed using a 1,024 channel matrix. Results were analyzed according to the Faedar Research program. APAAP procedures were performed as described by Cordell et al.12

Western blots were performed with cryopreserved cells according to the technique previously described.13 Briefly, lysates were prepared by solubilizing 10⁷ cells in 1 mL of a 10 mmol/L Tris HCl buffer, pH 7.8, containing 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 100 μmol/L EDTA. Fifty micrograms of proteins from each sample was separated by 12% SDS-polyacrylamide slab gel electrophoresis and electrophoretically transferred onto a nitrocellulose filter (Hybond-C nitrocellulose membrane; Amersham, Little Chaolt, UK). Immunodetection of the antigen was performed with the bc-l2/124 MoAb and a blotting detection kit for mouse antibodies (Amersham). Four AML samples with a variable number of bc-l2+ cells were studied. Negative control was performed using the Daudi cell line. Positive control was performed using the Val lymphoma line.

Surface marker analysis Surface markers were analyzed by flow cytometry as previously described with antibodies of the following specificities: CD13, CD14, CD15, CD33, and CD34. The staining was considered positive when 20% more cells than in the control were stained.

Liquid culture assay Ficoll-separated fresh cells (10⁷) at a concentration of 2 × 10⁶/mL were suspended in minimal essential medium (MEM) supplemented with 20% FCS and cultured in 75 cm² plastic flasks at 37°C in a humidified atmosphere containing 5% CO2. Cell cultures were maintained for 1 to 14 weeks, until a complete disappearance of cells was achieved. The content of each flask was removed every week centrifuged, and half of the supernatant (2.5 mL) was returned to the flask. The cells were resuspended in 2.5 mL of fresh medium. They were then counted and their viability was assessed by trypan blue exclusion test. They were then replaced into the original flask.

Leukemic progenitor (colony-forming unit-leukemia [CFU-L]) assay Ficoll-separated cells were plated at 10⁴/well (24 Multidish; Nunc, Rüski, Denmark) containing 0.5 mL Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 30% FCS (GIBCO, Paisley, UK), 0.8% methylcellulose (Methocel MC; Fluka, Buchs, Switzerland), and 12% conditioned medium of 5637 human bladder carcinoma cell line. Colonies (more than 40 cells) were counted under an inverse microscope after 7 days of incubation at 37°C in a humidified atmosphere with 5% CO2.

Statistical analysis The expression of bc-l2 was analyzed in two different ways. Whenever possible, the number of bc-l2+ cells was used as a quantitative parameter. In this instance, the relationships of the expression of bc-l2 to qualitative parameters (sex, FAB type, remission) were analyzed by the Student’s t-test. The relationships of the expression of bc-l2 to quantitative parameters (age, blood counts, percentage of marrow blasts, CFU-L number, and duration of culture) were studied by multiple regression analysis (a logarithmic transformation of white blood cell [WBC] number was performed). However, for a better illustration of the results (eg, to plot survival curves), it was sometimes necessary to define subgroups according to bc-l2 expression. A cutoff of 20% bc-l2+ cells was chosen to discriminate “bc-l2+” and “bc-l2-” 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.

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

RESULTS

Expression of bc-l2 in cell lines and leukemic cells. bc-l2 protein was detected by immunofluorescence in, respectively, 97% and 85% of KG1a and HL60 cell lines. The intensity of fluorescence was lower in HL60 than in KG1a cells. Daudi cells were negative, and were therefore used as negative controls for the Western blot.

The percentage of patient cells stained by anti–bc-l2 as assessed by flow cytometry and APAAP techniques were very well correlated (r = .97, P < 10⁻⁸). The results reported here will be those obtained by flow cytometry. The percentage of positive cells in each sample varied from 0% to 95%, with a median of 15% and a mean of 23%. The mean intensity of staining of positive cells was also variable between individual cases and was not correlated with the percentage of positive cells. Flow cytometry diagrams obtained for the KG1a line and two leukemic specimens are presented in Fig 1. The dis-
EXPRESSION OF bcl-2 PROTEIN IN AML

Fig 1. Flow cytometry analysis of bcl-2 protein expression in KG1a cell line (line A) and in two leukemia specimens (lines B and C). The left diagrams are obtained with control serum and the right diagrams with anti-bcl-2 MoAb. The respective percentages of bcl-2+ cells are: 96% (A), 78% (B), and 86% (C). The mean fluorescence intensities in positive cells (expressed in arbitrary units) are: 148 (A), 28 (B), and 52 (C). Histograms are designed with a scale of 256 channels using the Lysys program.

Distribution of cases according to the percentage of positive cells is given in Table 2. Twenty-eight of 82 samples (34%) contained 20% or more stained cells. Western blot confirmed the high level of expression of the 26-Kd protein in 4 cases (Fig 2).

There was no correlation of bcl-2 expression with sex, age, hemoglobin level, platelet level, and percentage of blasts in the marrow. However, the WBC count was significantly correlated with the percentage of bcl-2+ cells (P < .005) (Table 2).

The expression of bcl-2 according to FAB classification is presented in Table 3. The percentage of bcl-2+ cells was significantly higher in monoblastic (FAB M4 and M5) subtypes than in other subtypes (31% v 16%, P < .01). bcl-2 was negative (<10% cells stained) in all promyelocytic leukemias tested.

The expression of bcl-2 was compared with that of other surface markers. There was no correlation with CD13, CD14, CD15, or CD33. The mean percentage of bcl-2+ cells was significantly higher in CD34+ than in CD34− cases (29.5% v 14.6%, P < .01). This was confirmed by a significant correlation (P < .05) in linear regression test.

Correlations with leukemic cell cultures. The mean number of CFU-L recovered in semi-solid medium was not influenced by bcl-2 expression (mean number of CFU-L/10^5 cells plated ± SD: 104 ± 123 in bcl-2+ v 112 ± 128 in bcl-2− cases).

The behavior of leukemic cells in liquid cultures was assessed by counting the number of viable cells in culture every week and by the total duration of culture. The mean number of cells surviving in culture as assessed weekly is plotted in Fig 3 according to bcl-2 expression. At any week of culture, the number of surviving cells was higher in bcl-2+ than in bcl-2− cases.

The survival of blast cells in liquid culture was significantly correlated with the percentage of bcl-2+ cells (Table 2). The mean culture duration was 9.5 ± 3.6 weeks for leukemias

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Table 2. Correlations of bcl-2 Expression With Hematologic Characteristics and Complete Remission

<table>
<thead>
<tr>
<th>% bcl-2+ Cells</th>
<th>0-100 (all samples)</th>
<th>0-9</th>
<th>10-19</th>
<th>20-39</th>
<th>40-59</th>
<th>60-79</th>
<th>80-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>82</td>
<td>29</td>
<td>25</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Mean WBC (10^9/L)</td>
<td>63.9</td>
<td>35.4</td>
<td>54.3</td>
<td>85.9</td>
<td>78.2</td>
<td>162.2</td>
<td>94.8</td>
</tr>
<tr>
<td>Mean culture duration (wk)</td>
<td>5.7</td>
<td>2.9</td>
<td>4.1</td>
<td>6.6</td>
<td>12.0</td>
<td>11.2</td>
<td>13.6</td>
</tr>
<tr>
<td>Mean no. of CFU-L/10^6 cells</td>
<td>107</td>
<td>102</td>
<td>109</td>
<td>87</td>
<td>187</td>
<td>138</td>
<td>64</td>
</tr>
<tr>
<td>No. of patients in remission (%)</td>
<td>54 (66)</td>
<td>26 (97)</td>
<td>20 (80)</td>
<td>8 (64)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Percentage of bcl-2+ cells per sample as assessed by flow cytometry.
with 20% or more bcl-2+ cells versus 3.4 ± 1.9 weeks for cases with less than 20% stained cells \( (P < 10^{-3}) \). The culture also terminated more rapidly in M1 to M3 \((4.3 ± 3.2\text{ weeks})\) than in M4 to M5 \((7.1 ± 4.2\text{ weeks})\) FAB types \((P < .001)\), and in leukemias with hyperleukocytosis \((4.2 ± 3.1\text{ weeks})\) for cases with WBC \(≤30 \times 10^9/\text{L} \) \(v\) 6.9 ± 4.2 weeks for cases with WBC \(≥30 \times 10^9/\text{L} ; P < .005)\). Because of these associations, a multivariate analysis of the influence of percentage of bcl-2-stained cells, WBC, and FAB type on culture duration was performed. A highly significant correlation \((P < 10^{-5})\) was observed between the percentage of bcl-2+ cells and culture duration, but the other variables had no significant independent influence on this duration.

**Correlations with treatment outcome.** The overall CR rate was 54 of 82 (66%). The mean percentage of bcl-2-expressing cells was 11.1% in the samples from patients in whom remission was obtained versus 45.6% when remission was not obtained \((P < 10^{-3})\). The CR rate decreased as the percentage of bcl-2+ cells increased, as is shown in Table 2, and none of the 16 patients whose percentage of bcl-2-expressing cells exceeded 60% went into remission. Overall, only 8 of 28 \((29\%)\) bcl-2+ versus 46 of 54 \((85\%)\) bcl-2- cases achieved remission \((P < 10^{-3})\). The main cause for not achieving remission was resistant disease \((14\text{ of } 28\text{ bcl-2+ cases } v \ 5\text{ of } 54\text{ bcl-2-}, P < 10^{-3})\). CR was also highly correlated with survival of blast cells in culture (mean culture duration: 3.5 weeks for patients in CR \(v\) 9.8 weeks for patients not in CR \(P < 10^{-5}\)). Other factors influencing remission were age (CR rate: 34 of 43 \((79\%)\) for patients aged 60 or less \( v\) 20 of 40 \((50\%)\) for patients older than 60, \(P < .01)\), WBC counts (CR rate: 23 of 44 \((43\%)\) for cases with WBC \(≤30 \times 10^9/\text{L} \) \(v\) 31 of 38 \((82\%)\) when WBC were \(<30 \times 10^9/\text{L}, P < .001)\), and CD34 expression (CR rate: 22 of 46 \((48\%)\) for CD34+ cases \(v\) 32 of 36 \((89\%)\) for CD34- cases, \(P < 10^{-3})\). There was no significant influence of other parameters studied, including FAB subtype.

In univariate analysis, survival was significantly influenced by several factors: age \((≤60 \text{ years } v >60 \text{ years}, P < .01)\), CD15 expression \((≥20\% \ v≤20\%, P = .01)\), CD34 expression \(<20\% \ v≥20\%, P < .005)\), bcl-2 expression \(<20\% \ v≥20\%, P < .005)\), and culture duration \((≤5 \text{ weeks } v >5 \text{ weeks}, P < 10^{-3})\). The Kaplan-Meier plot of survival duration of patients with less than 20% or \(≥20\%\) bcl-2+ cells is given in Fig 4. There was no significant effect of other parameters, including FAB type and WBC. The respective effects of these parameters were tested by multivariate analysis. Culture duration \((P < 10^{-5})\), age \((P < 10^{-4})\), CD34 \((P < .01)\), and CD15 \((P < .05)\) were significantly associated with survival. bcl-2 expression was not significant. However, in step-by-step analysis, bcl-2 became the most significant factor \((P < 10^{-5})\) when survival of blasts in culture was removed from the model. Step-by-step analysis otherwise did not significantly modify the results of the multivariate analysis.

Factors influencing survival were tested for their influence on CR duration in 54 patients. In univariate analysis, only culture duration had a borderline influence \((P = .05)\), bcl-2 expression was not significant. In multivariate analysis, no significant factor could be detected. However, the heterogeneity of postinduction treatments (mainly according to age) and the fact that in some categories there were very few pa-
tients in remission makes it difficult to draw a conclusion about remission duration.

DISCUSSION

The translocation of the BCL-2 gene to the Ig heavy chain gene in the t(14;18) abnormality appears to play a key role in the pathogenesis of follicular lymphoma, resulting in the overexpression of a chimeric gene. High levels of nontranslocated BCL-2 gene products have also been observed in other lymphoid cell malignancies. In this report, we present evidence that bcl-2 protein is expressed in a high proportion of AML. Western blot analysis confirmed that the 26-Kd bcl-2 protein was detected. These findings are consistent with previous immunocytologic studies showing the presence of bcl-2 in normal BM, in which it can be detected in immature myeloid cells up to the stages of promyelocyte and erythroblast. Moreover, Delia et al recently reported on bcl-2 protein and mRNA expression in CD34+ normal hematopoietic cells and in myeloid neoplasias. Myeloblastic cell lines and 11 of 14 AML cases tested were bcl-2+. Our study performed mainly by flow cytometry showed that the binding of the anti–bcl-2 MoAb to leukemic cells was heterogeneous (from 0% to 95% of cells), which makes it difficult to define two groups, one positive and one negative. We arbitrarily chose to consider AML with 20% or more stained cells as positive when it was necessary for statistical analysis to design two categories, but it is likely that some cases with less than 20% positive cells would be considered positive by other techniques such as Western blot. However, the use of flow cytometry allowed us to quantify easily the expression of bcl-2 and to show a continuous association between the number of bcl-2+ cells and other parameters such as WBC or treatment outcome.

We found a significant correlation between bcl-2 expression and WBC, and between bcl-2 and monoblastic subtype, which is consistent with the fact that AML of FAB type M4 and M5 usually present with higher WBC. bcl-2 was also more frequently detected in the immature forms (M1 and M2) of the granulocytic leukemias than in the more differentiated M3 subtype. We found bcl-2 to be positive in the HL-60 promyelocytic line. However, the intensity of fluorescence was much lower than in the more immature KG1a line, as reported by Delia et al. Moreover, these investigators showed that differentiation of HL-60 resulted in a loss of bcl-2 expression. These data obtained with AML cells and cell line are similar to those reported on normal marrow and suggest that the high expression of bcl-2 in leukemia cells is a consequence of the absence of differentiation rather than of deregulated expression.

bcl-2 appears to prolong lymphoid cell survival by blocking programmed cell death. Moreover, the transfection of BCL-2 gene into the myeloid line FDC-P1 prolongs the cell survival in growth factor-deprived medium. We therefore investigated the survival of blasts maintained in liquid culture in the absence of growth factor. The duration of blast survival was very well correlated with the percentage of bcl-2-expressing cells. Although the difference is less dramatic, there was also an increased cell survival in monoblastic subtypes, and in cases with higher WBC counts. However, in multivariate analysis, bcl-2 was the only factor that predicted culture duration. Therefore, it is likely that leukemias with monoblastic subtypes and hyperleukocytosis were associated with longer culture duration because they contain more bcl-2+ cells. We also observed a moderate increase (about threefold) of the number of cells in suspension during the first week of culture. However, the fact that the frequency of clonogenic cells was not correlated with the percentage of bcl-2+ cells suggests that the advantage in survival was mainly due to delayed cell death and not to increased proliferating capacities. These results obtained with fresh leukemic cells are thus similar to those obtained on bcl-2–transfected hematopoietic cell lines.

The patient outcome after intensive chemotherapy was highly predicted by bcl-2 expression. All patients reported in this series were treated by intensive chemotherapy. The percentage of bcl-2+ cells was significantly higher in patients who did not achieve remission, and none of the 16 patients with leukemic samples containing more than 60% bcl-2+ cells went into remission. Resistance to treatment was the main cause of failure. As Tsujimoto reported that transfected hematopoietic lines exhibit higher resistance to stresses such as heat shock, ethanol, or methotrexate, it may be hypothesized that bcl-2 is implicated in resistance to chemotherapy, although other mechanisms such as multidrug resistance (MDR1) have also been described in leukemic patients, particularly those with CD34+ leukemias. Patients with bcl-2+ leukemias also had a shorter survival. In multivariate analysis, bcl-2 expression was the most important predictive factor, before other known parameters such as age and CD34 expression, which were independently associated with survival. If blast cell behavior in culture was taken into account, the value of bcl-2 as an independent predictive factor was lost, which indicates that both factors were highly correlated. Our findings confirm the already reported prognostic value of AML cells in liquid culture and give a biologic basis to this phenomenon.

In conclusion, we show that bcl-2 is expressed on AML cells, and correlates with biologic findings such as hyperleukocytosis, monoblastic types, and blast behavior in liquid culture. Moreover, this expression is associated with very poor prognosis and may implicate modified therapeutic strategies in bcl-2+ patients.

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