Clinical Relevance of BCL-2 Overexpression in Childhood Acute Lymphoblastic Leukemia

By Elaine Coustan-Smith, Akira Kitanaka, Ching-Hon Pui, Lisa McNinch, William E. Evans, Susana C. Raimendi, Frederick G. Behm, Maurizio Arico, and Dario Campana

Enforced BCL-2 gene expression in leukemic cell lines suppresses apoptosis and confers resistance to anticancer drugs, but the clinical significance of increased BCL-2 protein levels in acute lymphoblastic leukemia (ALL) is unknown. Among 52 children with newly diagnosed ALL, BCL-2 expression in leukemic lymphoblasts ranged widely, from 4,464 to 59,753 molecules of equivalent soluble fluorochrome per cell (MESF), as determined by flow cytometry. The mean (±SD) level of MESF in 43 cases of B-lineage ALL (19,410 ± 11,834) was higher than that detected in CD10+ B-lymphoid progenitors from normal bone marrow (460 ± 314; P < .001), and CD19+ peripheral blood B lymphocytes (7,617 ± 1,731; P = .02). Levels of BCL-2 in T-ALL cases (17,909 ± 18,691) were also generally higher than those found in normal CD1a+ thymocytes (1,762 ± 670), or in peripheral blood T lymphocytes (9,687 ± 3,019). Although higher levels of BCL-2 corresponded to higher leukemic cell recoveries after culture in serum-free medium, they did not correlate with higher cell recoveries after culture on stromal layers, or with in vitro resistance to vincristine, dexamethasone, 6-thioguanine, cytarabine, teniposide, daunorubicin or methotrexate. BCL-2 protein levels did not correlate with presenting clinical features. Unexpectedly, however, lower-than-median MESF values were significantly associated with the presence of chromosomal translocations (P = .010). Notably, all six cases with the Philadelphia chromosome, a known high-risk feature, had low levels of BCL-2 expression (P = .022). Higher levels of BCL-2 were not associated with poorer responses to therapy among 33 uniformly treated patients, and were not observed in three patients studied at relapse. In conclusion, increased BCL-2 expression in childhood ALL appears to enhance the ability of lymphoblasts to survive without essential trophic factors, and is inversely related to the presence of chromosomal translocations. However, it does not reflect increased disease aggressiveness or resistance to chemotherapy.

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The product of the BCL-2 gene suppresses programmed cell death (apoptosis). Thus, enforced BCL-2 expression delays apoptosis of cell lines deprived of essential survival factors, and prolongs the life-span of lymphocytes in BCL-2 transgenic mice. Overexpression of BCL-2 may also confer resistance to cytotoxic agents. For example, enforced BCL-2 expression increases the resistance of lymphocytes to glucocorticoids and radiation, and of leukemic cell lines to a variety of anticancer drugs. Conversely, cytokine- or antisense-mediated reduction of BCL-2 expression enhances sensitivity to several cytotoxic drugs. These findings indicate that BCL-2 could function as a multidrug resistance molecule and that its measurement could be of prognostic value. This hypothesis gains support from the association of higher BCL-2 levels with poorer responses to chemotherapy in acute myeloid leukemia (AML),1,12 with unfavorable histology and N-MYC amplification in neuroblastoma,13 with androgen independence in prostate carcinoma, and with the occurrence of lymph node metastasis in breast carcinoma.

We have found that B-lineage acute lymphoblastic leukemia (ALL) cells accumulate high levels of BCL-2 protein, compared with their normal counterparts (B-cell progenitors of the bone marrow [BM]), and that BCL-2 expression varies markedly among cases. In the study reported here, we tested whether cellular levels of BCL-2 in leukemic lymphoblasts correlate with (1) in vitro growth capacity in cultures supported by BM-derived stromal layers, (2) resistance to apoptosis in cultures without stromal layers, (3) sensitivity to antileukemic drugs in vitro, and (4) the presenting clinico-biologic features and event-free survival (EFS) of patients enrolled in a single program of chemotherapy.

MATERIALS AND METHODS

Cells. BM samples were taken from healthy BM transplantation donors, aged 7 to 23 years (median, 15), and peripheral blood (PB) samples from healthy adult volunteers. Thymic glands were from children undergoing cardiac surgery; cell suspensions were prepared with forceps and surgical blades. Leukemic BM samples were collected at diagnosis from 52 children with newly diagnosed ALL, aged less than 1 to 18 years (median, 5); three patients were also studied at the time of first relapse. In 43 cases, greater than 80% of the blasts expressed CD19, CD22, and HLA class II antigens, but lacked surface Igs and were classified as B-lineage ALL. In the remaining nine cases, lymphoblasts expressed cytoplasmic or surface CD3, CD7, CD5, and CD2, and were classified as T-lineage ALL. All samples were collected with the informed consent of the patients or their guardians, and with approval from the St Jude Institutional Review Board. Some of the samples investigated had already been included in our previous study. Mononucleated cells were collected after centrifugation on a density gradient (Lymphoprep, Nycomed, Oslo, Norway) and washed three times in phosphate-buffered saline (PBS). Leukemic cell lines 380, Nalm6, OP-1, and RS4;11, were available in our cell bank.
Flow cytometric estimates of BCL-2 protein expression. To detect BCL-2, we used a monoclonal antibody (MoAb) that recognizes the 26-kD BCL-2α protein (Dako, Carpinteria, CA). Cell staining with this antibody is limited to the cytoplasm and is completely abrogated by preincubation with the synthetic peptide GAAPAP-GIFSSQPG, analogous to that used for immunization. After fixing and permeabilizing mononucleated cells with 0.25% para-formaldehyde and 0.2% Tween 20, we added anti-BCL-2 MoAb (IgG1 class), followed by goat-antimouse Ig or IgG1 conjugated to fluorescein isothiocyanate (FITC; Jackson Immunoresearch Laboratories Inc, West Grove, PA), as previously described. For double-staining experiments, we used anti-BCL-2 in combination with CD10 (IgM; Boehringer Mannheim, Indianapolis, IN), CD19 (Bly3, IgM; gift of Dr S. Poppema, Edmonton, Canada), or CD1a (IgG2b; Becton Dickinson, San Jose, CA), followed by isotype-specific goat-antimouse antisera conjugated to phycoerythrin (from Jackson Immunoresearch Laboratories and Southern Biotechnology Associates, Birmingham, AL). Cells were analyzed with a FACScan flow cytometer with Lysys II software (Becton Dickinson). The fluorescence intensity of BCL-2 was measured with detectors and amplifiers set on a logarithmic scale, and a geometric calculation mode. The channel values were converted into the number of molecules of equivalent soluble fluorochrome per cell (MESF) by comparing the results of anti-BCL-2 staining with calibrated fluorescence reference standards (Quantum Series; Flow Cytometry Standards Corp, San Juan, Puerto Rico), which were run in parallel with the samples, as previously described. Fitting of sample measurements on the standard curve was done using the GRAPH software (MicroMath, Salt Lake City, UT). Fluorescence intensity achieved with an isotype matched unreactive antibody was subtracted from that obtained with anti-BCL-2 to calculate the net MESF values. The calculation mode (geometric) used in this study for the log to linear conversion was different than that used in our previous study (arithmetic). This resulted in MESF values lower than previously reported. The consistency of BCL-2 measurements was tested periodically in normal lymphocytes and in leukemic cells. In PB samples from two donors tested three and five times over a 2-year period, the coefficient of variation (CV) of BCL-2 measurements was 0.17 and 0.078. In repeated testing of two cell lines (380 and Nalm6) and two ALL samples, the CV ranged from 0.006 to 0.10 (median 0.004). Western blotting. Mononucleated cells (> 90%) from ALL samples and cell lines were lysed at 4°C in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mg/mL aprotinin, and 1 mmol/L EDTA). Cell lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a nitrocellulose membrane (Hybond-ECL, Amersham, Arlington Heights, IL), which then was incubated with 5% dehydrated milk in TBST buffer (20 mmol/L Tris-HCl [pH 7.6], 137 mmol/L NaCl, 0.1% Tween 20) for 2 hours. After incubation with anti-BCL-2 MoAb for 2 hours at 20°C, the membrane was washed three times with TBST, and incubated with horseradish peroxidase-conjugated antimouse IgG (Amersham) for 1 hour. After further washing, the membrane was incubated with the enhanced chemiluminescence substrate solution, and exposed to Kodak XAR-2 film (Eastman Kodak, Rochester, NY). In vitro culture of ALL cells. Leukemic lymphoblasts were cultured in the presence or absence of the syngonal layers, as previously described. Briefly, BM stromal cells, obtained from healthy BM donors, were prepared in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA), using RPMI-1640 (Whittaker Bioproducts Inc, Walkersville, MD) with 10% fetal calf serum (FCS; Whittaker) and 10−8 mol/L hydrocortisone (Sigma, St Louis, MO) to feed cells until confluent layers had formed. Residual T cells were eliminated by treatment with a CD3 MoAb (T10B9) and rabbit complement. Before each experiment, the media from cultures of BM stroma and washed the adherent cells seven times with AIM-V medium-serum free (GIBCO, Grand Island, NY). Leukemic cells were resuspended in AIM-V medium at a final concentration of 1.5 × 106/mL, after T cells had been depleted with CD4- and CD8-conjugated immunomagnetic beads (Dynal, Oslo, Norway). Two hundred microliters of the suspension was then placed in the wells of a 96-well flat-bottomed tissue culture plate or seeded onto marrow stromal cells in identical plates. Cells were cultured for 7 days in an incubator set at 37°C, 5% CO2 and 90% humidity. At the termination of cultures, viable CD19+ lymphoblasts were counted with a FACScan flow cytometer with Lysys II software (Becton Dickinson), as previously described. The percentage of viable CD19+ lymphoblasts recovered was calculated with the formula: (no. of CD19+ lymphoblasts after 7 days of culture) × 100/(no. of CD19+ lymphoblasts after 1 hour of culture). Results are reported as the means of duplicate experiments.

Determination of drug sensitivity in vitro. The in vitro drug sensitivity of ALL cells was determined with a stroma-supported immunocytometric assay, as previously described. Briefly, BM stromal layers were prepared in 96-well U-bottom microtiter plates, as described above. ALL cells were resuspended in AIM-V medium and placed in the wells of the plates onto BM stromal cells. Blast cells from individual patients were tested for sensitivity to each of seven antileukemic drugs over a range of concentrations: vincristine sulphate (0.0009 to 0.5 μmol/L), dexamethasone (0.001 to 10 μmol/L), daunorubicin (0.003 to 0.35 μmol/L), 6-thioguanine (0.47 to 120 μmol/L), methotrexate (0.01 to 25 μmol/L), and cytosine-β-D-arabinofuranoside (0.032 to 20 μmol/L), all from Sigma (St Louis, MO); and teniposide (0.04 to 25 μmol/L; Bristol Myers Squibb, Wallingford, CT). Two wells were used for each drug concentration, and four wells contained untreated leukemic cells. All cell cultures were incubated for 4 days at 37°C, 5% CO2 and 90% humidity. At termination of the cultures, cells were stained with antibodies and counted by flow cytometry, as described above. The concentration of antileukemic agent that was lethal to 50% of the blast cells (LC50) was determined for each patient by fitting the percent viability versus drug concentration data to a sigmoid model, using ALLFIT software from the National Institutes of Health.

Statistical analysis. Differences in BCL-2 expression between different cell populations were analyzed by Student’s t-test. Distributions of cell survival in culture, cytotoxic drug LC50 and commonly measured presenting features, according to cellular BCL-2 expression (above or below the median MESF value), were compared by Fisher’s exact test. The probability of surviving without an adverse event (ie, failure to enter remission, relapse, or death due to any cause) was estimated by the method of Kaplan and Meier, applied to follow-up observations to February 1, 1995. Comparisons of EFS distributions were made by the exact logrank test and stepwise Cox regression analysis, and were limited to 33 patients who had been treated uniformly in Total Therapy Study XII (median follow-up time, 46 months).

RESULTS

BCL-2 expression in normal and leukemic lymphoid cells. We first calculated BCL-2 expression in 52 cases of childhood ALL, comparing mean fluorescence intensities obtained with the anti-BCL-2 antibody to values obtained with beads of calibrated fluorescence. In all 52 cases, greater than 80% of the blasts had BCL-2 fluorescence levels above the highest achieved with the nonreactive control antibody. The
Mean (±SD) value in 43 cases of B-lineage ALL was 19,410 ± 11,834 MESF, a significantly higher value than that found among highly proliferating CD10+ B-cell precursors from normal BM (450 ± 314; n = 6; P < .001; Fig 1) or normal PB CD19+ B lymphocytes (7,617 ± 1,731; n = 6; P < .02). The degree of BCL-2 expression in the 43 B-lineage ALL cases did not correlate with that of CD22, a B-cell marker preferentially expressed in the cytoplasm of leukemic B-lymphoid cells (r² = .25), indicating that the heterogeneity of BCL-2 fluorescence intensities was not related to differences in cellular susceptibility to permeabilization.

Levels of BCL-2 expression in nine cases of T-ALL (17,909 ± 18,691) were higher than those in immature CDla+ cells from three infant thymic glands (1,762 ± 670; Fig 1) and in PB T lymphocytes (9,687 ± 3,019; n = 6). Although not achieving statistical significance, these differences, nonetheless, indicate that asynchronous upregulation of BCL-2 protein expression occurs in both B- and T-lineage ALL.

To determine the specificity of the anti-BCL-2 antibody and to ensure that heterogeneous staining in ALL samples was exclusively caused by variations in BCL-2 protein levels, and was not influenced by cross-reactivity of the antibody with other molecules, we performed Western blotting analysis in 3 B-lineage ALL cases with different karyotypes, 1 T-ALL case, and 4 ALL cell lines, including 1 (380) with the t(14; 18) translocation. In all samples, only the expected 26-kD band was seen (Fig 2).

Higher levels of BCL-2 expression correlate with resistance to apoptosis under unfavorable in vitro culture conditions. BM-derived stromal layers inhibit cell death by apoptosis in cases of B-lineage ALL.19 Thus, we determined the relation between levels of BCL-2 expression and the cells’ ability to survive in culture with and without stroma. Among 26 cases of B-lineage ALL studied, 20 had virtually no viable CD19+ lymphoblasts (±5% of those originally seeded) after 7 days of culture in serum-free medium without stroma, whereas six had a leukemic cell recovery ranging from 18% to 90% (median, 58%). BCL-2 expression above the median value of 16,650 MESF was found in all 6 cases with higher cell recovery but in only 7 of the 20 with lower recoveries (P = .015; Table 1); however, BCL-2 levels were not linearly related to the percentage of cell recovery (data not shown). Expression of the CD22 control was not associated with the percentage of cell recovery after culture: MESF values above the median were observed in 3 of the 6 cases with higher cell recovery, and in 10 of the 20 with lower recoveries.

In parallel experiments, the recovery of B-lineage ALL cells from stromal layers after 7 days of culture ranged from 4% to 183% (median, 105%) of those originally seeded. There was no apparent relation between BCL-2 expression and the percentage of CD19+ lymphoblasts recovered after 7 days of culture on stromal layers relative to the number recovered after 1 hour. Table 1. Relation of BCL-2 Expression to Cell Recovery After Culture

<table>
<thead>
<tr>
<th>Culture Condition</th>
<th>% Cell Recovery</th>
<th>BCL-2 Expression†</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stroma</td>
<td>&lt;5</td>
<td>Low 13</td>
<td>High 7</td>
</tr>
<tr>
<td></td>
<td>&gt;5</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>With stroma</td>
<td>&lt;105</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>&gt;105</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 1. Relation of BCL-2 Expression to Cell Recovery After Culture

* Percentage of CD19+ lymphoblasts recovered after 7 days of culture on stromal layers relative to the number recovered after 1 hour.
† Reported as values below (Low) or above (High) median value of 16,650 MESF.
‡ By Fisher’s exact test.
sensitivity to cytotoxic drugs in vitro. BCL-2 protein levels were measured in 18 cases by flow cytometry and expressed as MESF (see Materials and Methods). Values are represented as mean ± SD; cases with LC50 above (□) or below (□□) median for each cytotoxic drug are compared. AraC, cytarabine; MTX, methotrexate; VCR, vincristine; VM26, teniposide; 6-TG, 6-thioguanine; DNR, daunorubicin; DEX, dexamethasone.

and cell recovery rates, in sharp contrast to results obtained without stroma (Table 1). Thus, levels of BCL-2 expression appear to influence resistance to trophic factor deprivation but do not affect the ability of leukemic lymphoblasts to survive and proliferate in cultures simulating physiologic conditions.

BCL-2 expression and drug sensitivity in vitro. To assess the relationship between expression of BCL-2 and sensitivity to several commonly used antileukemic drugs in vitro, we used an assay in which cell viability is maintained by BM stromal layers. Among the 18 cases studied, considerable variability was observed for each drug. The LC50 ranged from 0.007 to >0.5 μmol/L (median, 0.05 μmol/L) for vincristine, 0.004 to 0.04 μmol/L (median, 0.01 μmol/L) for dexamethasone, <0.003 to >0.35 μmol/L (median, 0.01 μmol/L) for daunorubicin, <0.5 to >120 μmol/L (median, 5.15 μmol/L) for 6-thioguanine, <0.01 to >25 μmol/L (median, 0.04 μmol/L) for methotrexate, <0.032 to >0.35 μmol/L (median, 0.17 μmol/L) for cytarabine, and 0.05 to 2.5 μmol/L (median, 0.33 μmol/L) for teniposide. Despite this marked variability among individual patients, levels of BCL-2 expression were not correlated with in vitro drug sensitivity for any of the seven antileukemic agents tested (Fig 3).

Relation of BCL-2 expression to clinicobiologic features and treatment outcome in childhood ALL. Cellular levels of BCL-2 were not significantly related to the presenting leukocyte count, age, race, serum lactate dehydrogenase levels, presence of central nervous system (CNS) involvement, the percentage of cells in S phase, immunophenotype, or leukemic cell ploidy (Table 2). However, BCL-2 expression was inversely related to the presence of chromosomal translocations. That is, translocations were present in 18 of the 25 patients with MESF values below the median, compared to 8 of 24 with higher levels of BCL-2 expression (P = .010; Table 2). Notably, all six cases with the t(9;22)(q34;q11) (*“Philadelphia-chromosome”), a known high-risk feature,26 had low BCL-2 expression (P = .022).

EFS at 3 years was 81% ± 11% (SE) for the 16 patients with higher BCL-2 levels and 41% ± 13% for the 17 with lower values (P = .04; Fig 4). Three of the 33 patients (two in the lower BCL-2 group) did not achieve complete remission. Among other presenting features listed in Table 2, only age <1 or >10 years was significantly associated with poorer outcome (P = .001 by univariate logrank test). When all factors were considered in a stepwise Cox regression analysis, only age entered the model as an independent significant prognostic factor (P < .01).

The poor prognosis of the patients with lower BCL-2 levels could have been attributed to the inclusion of seven patients with high-risk karyotypic features [ie, t(9;22) and 11q23 abnormalities], five of whom have already had an adverse event. None of the cases with unfavorable karyotypes were in the high BCL-2 group. When EFS analysis was limited to the 26 patients without unfavorable karyotypes, differences in outcome according to BCL-2 expression were not significant (P = .1371).

Table 2. Relation of BCL-2 Expression to Commonly Measured Presenting Characteristics of ALL Patients

<table>
<thead>
<tr>
<th>Feature</th>
<th>Category</th>
<th>Low</th>
<th>High</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal translocation</td>
<td>No</td>
<td>7</td>
<td>16</td>
<td>.010</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>18</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Philadelphia chromosome</td>
<td>No</td>
<td>19</td>
<td>24</td>
<td>.022</td>
</tr>
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<td></td>
<td>Yes</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ploidy</td>
<td>51-67 chromosomes</td>
<td>3</td>
<td>8</td>
<td>.095</td>
</tr>
<tr>
<td></td>
<td>Others</td>
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<td>16</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td>Black</td>
<td>4</td>
<td>0</td>
<td>.110</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>22</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>7</td>
<td>12</td>
<td>.249</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>19</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>1-10</td>
<td>13</td>
<td>17</td>
<td>.400</td>
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<tr>
<td></td>
<td>&lt;1 or &gt;10</td>
<td>13</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Leukocyte count (×10^9/L)</td>
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<td>10</td>
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<td>.404</td>
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<tr>
<td></td>
<td>&gt;50</td>
<td>16</td>
<td>12</td>
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</tr>
<tr>
<td>Immunophenotype</td>
<td>B-lineage</td>
<td>20</td>
<td>23</td>
<td>.465</td>
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<tr>
<td></td>
<td>T-lineage</td>
<td>6</td>
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<td></td>
</tr>
<tr>
<td>CNS involvement</td>
<td>No</td>
<td>18</td>
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<td>.699</td>
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<tr>
<td></td>
<td>Yes</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Serum LDH (U/L)</td>
<td>=770</td>
<td>13</td>
<td>13</td>
<td>.767</td>
</tr>
<tr>
<td></td>
<td>&gt;770</td>
<td>10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Percent S-phase</td>
<td>≤6.4</td>
<td>11</td>
<td>9</td>
<td>.99</td>
</tr>
<tr>
<td></td>
<td>&gt;6.4</td>
<td>14</td>
<td>10</td>
<td></td>
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</tbody>
</table>

Abbreviation: LDH, lactate dehydrogenase.
* Reported as values below (Low) or above (High) median value of 17,203 MESF.
† By Fisher’s exact test.
‡ Data not available for all cases.
BCL-2 expression was tested sequentially in three cases at diagnosis and at relapse. In each instance, leukemic lymphoblasts at relapse had slightly lower BCL-2 expression than at diagnosis, indicating that the emergence of drug-resistant clones is not associated with an increase in BCL-2 expression (Table 3).

**DISCUSSION**

In this study, we show that BCL-2 expression in B-lineage and T-lineage ALL is not only markedly higher than that of normal lymphoid progenitors, but it also generally surpasses the levels of expression seen in mature resting B and T lymphocytes. Cellular levels of BCL-2 were not related to the response of leukemic lymphoblasts to cytotoxic drugs in vitro or in vivo. However, higher BCL-2 levels were associated with improved ability of the cells to survive under a very unfavorable culture conditions (in the absence of stroma-derived growth factors), a finding that parallels observations with factor-dependent cell lines. Thus, the influence of BCL-2 expression on cellular susceptibility to apoptosis appears to depend on the cause of cell death.

Abnormally high levels of BCL-2 at critical stages of differentiation may also be involved in oncogenesis. A paradigm of BCL-2 participation in the development of neoplasia is follicular B-cell non-Hodgkin's lymphoma (NHL), where cells exhibit high levels of BCL-2 resulting from the t(14;18) translocation, which brings the BCL-2 gene on chromosome 18 next to the Ig heavy chain locus (14q32). The molecular events that lead to BCL-2 upregulation in ALL are unknown but do not appear to involve rearrangements or duplications of the BCL-2 gene. Incomplete Ig or T-cell receptor rearrangements, which result in an arrest of differentiation, are consistently found in ALL. We speculate that early leukemogenic events should protect lymphoid progenitors with defective rearrangements from apoptosis, perhaps through untimely upregulation of BCL-2. An intriguing observation in our study was the significantly lower BCL-2 protein expression in cases with chromosomal translocations, including the t(9;22). It may well be that in these cases molecular events other than BCL-2 upregulation, such as expression of BCR-ABL, are sufficient to prolong the life-span of effete cells with nonfunctional antigen-receptor gene rearrangements. In this regard, it is relevant that an inverse association between BCL-2 expression and p53 abnormalities (which inhibit apoptosis) has been observed in breast carcinoma.

The lack of association between higher BCL-2 expression and resistance to cytotoxic drugs does not support the hypothesis raised by studies with model cell lines, in which modulation of BCL-2 levels strongly influenced susceptibility to apoptosis induced by anticancer drugs. This apparent discrepancy is unlikely to be caused by lower levels of BCL-2 expression in leukemic cells from patients, compared with those in BCL-2-transfected cell lines, for such cell lines have comparable levels of expression to those found in primary cells (E.C.-S., D.C., unpublished results, November 1994). Rather, the antiapoptotic effects of BCL-2 overexpression in ALL may be offset by the activity of other molecules. BCL-2 activity requires formation of heterodimers with the related protein BAX, which antagonizes BCL-2 function and accelerates apoptosis. Thus, the equilibrium in the formation of BCL-2:BAX heterodimers (suppressors of death) and BAX:BAX homodimers (activators of death) appears to be central in the molecular regulation of apoptosis. In addition, several other BCL-2-related family members, which also regulate apoptosis have recently been identified. These include the suppressors of death BCL-XL, MCL-1, A1, and BAG, and additional death inducers BCL-Xs (a spliced version of BCL-XL), BAD, and BAK. Conceivably, a balance between the activity of all these molecules ultimately determines the cells' susceptibility to apoptosis.

Previous studies of BCL-2 expression in clinical samples have yielded contrasting results. Strong expression of BCL-2 was reported to be associated with unfavorable prognostic factors and poorer responses to therapy in AML, neuroblastoma, prostate carcinoma, and breast carcinoma; no correlations were found in high-grade NHL, and an inverse correlation was observed in small cell lung carcinoma and breast carcinoma (ie, better outcomes were seen in cases with lower or undetectable BCL-2 expression). A recent study of ALL failed to disclose any correlation between BCL-2 expression and outcome; however, these data may not be adequate, as BCL-2 evaluation was done by immuno-histochemistry, which does not afford precise and objective quantitation of antigen expression, especially when one considers the biological paradox that early leukemogenic events should protect lymphoid progenitors with defective rearrangements from apoptosis, perhaps through untimely upregulation of BCL-2.

**Table 3. BCL-2 Expression at Diagnosis and at Relapse**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Relapse*</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>9,964</td>
<td>8,193</td>
</tr>
<tr>
<td>2</td>
<td>12,992</td>
<td>11,663</td>
</tr>
<tr>
<td>3</td>
<td>21,963</td>
<td>16,603</td>
</tr>
</tbody>
</table>

Reported as MESF.

* Relapses occurred 9, 34, and 39 months after diagnosis, respectively.
siders that virtually all ALL cases express BCL-2. Nonetheless, our study clearly shows that strong BCL-2 expression does not confer a poor prognosis. On the contrary, lower expression did not emerge as an independent prognostic indicator, though in this relatively small series of patients BCL-2 expression did not emerge as an independent prognostic indicator, this observation warrants further studies to clarify its prognostic importance.

Griffiths et al. observed that murine B-cell precursors, like their human equivalents, are ultrasensitive to apoptosis and express low levels of BCL-2. They speculated that reten-

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poietic cell survival and cooperates with c-myc to immortalize pre-
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