Prolonged Survival of B-Lineage Acute Lymphoblastic Leukemia Cells Is Accompanied by Overexpression of bcl-2 Protein

By Dario Campana, Elaine Coustan-Smith, Atsushi Manabe, Michael Buschle, Susana C. Raimondi, Frederick G. Behm, Richard Ashmun, Maurizio Aricò, Andrea Biondi, and Ching-Hon Pui

Overexpression of bcl-2 delays the onset of apoptosis in lymphohematopoietic cells. We measured levels of bcl-2 protein in normal and leukemic human B-cell progenitors with a specific monoclonal antibody and flow cytometry. Normal immature B cells had low levels of bcl-2 protein; the intensity of fluorescence, expressed as molecules of soluble fluorochrome per cell, within CD10+ cells was 3,460 ± 1.050 (mean ± SD; 5 samples). In 16 cases of B-lineage acute lymphoblastic leukemia (ALL), cells had levels of bcl-2 that were strikingly higher than those of their normal counterparts (33,560 ± 14,570; P < .001 by t-test analysis). We next investigated whether the intensity of bcl-2 expression correlated with the resistance of immature B cells to in vitro culture. In 12 cases of B-lineage ALL, the cells recovered after 7 days of culture on allogeneic bone marrow stromal layers were 69% to 178% (median, 95.5%) of those originally seeded. Prolonged survival of leukemic cells in vitro was observed even in the absence of stromal layers in 6 of these 12 cases; the intensity of bcl-2 protein expression in these cases was 45,000 ± 13,270, compared with 21,500 ± 7,260 in the 6 cases in which greater than 99.5% of cells rapidly died by apoptosis under the same culture conditions (P = .003). Five immature B-cell lines, continuously growing in the absence of stroma, had the highest bcl-2 expression (79,400 ± 20,330). By contrast, most normal CD19+, slg- immature B cells died despite the presence of bone marrow stromal layers; 9.7% to 28.2% were recovered after 7 days of culture in three experiments. We conclude that abnormal bcl-2 gene expression influences the survival ability of B-cell progenitors. This may contribute to leukemogenesis and explain the aptitude of leukemic lymphoblasts to expand outside the bone marrow microenvironment.

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The product of the human gene bcl-2 antagonizes the triggering of programmed cell death ("apoptosis"). Thus, overexpression of bcl-2 delays apoptosis in factor-dependent cell lines cultured under suboptimal conditions and in cortical thymocytes exposed to glucocorticoid, radiation, and CD3 antibodies.

It has been postulated that abnormally high levels of bcl-2 at critical stages of differentiation may favor the development of neoplasia. This concept is supported by the observation of discrepancies in bcl-2 protein expression between follicular B-cell non-Hodgkin’s lymphoma (NHL) cells, which exhibit high levels of bcl-2, and their normal counterparts, proliferating germinal center cells, which appear to be bcl-2 negative and are susceptible to apoptosis during the selection processes that occur during immune response in the peripheral lymphoid organs. Abnormally high expression of bcl-2 by these cells may suppress apoptosis, leading to the survival of inappropriate clones. In NHL, increased bcl-2 synthesis results from a chromosomal translocation, t(14;18), that brings the bcl-2 gene on chromosome 18 at q21 next to the Ig heavy chain locus (14q32). However, abnormally high levels of the bcl-2 protein have also been observed in rare cases of NHL that lack either karyotypic or molecular evidence of bcl-2 gene rearrangement, suggesting that other mechanisms may induce overexpression of bcl-2.

Whether malignancies beyond NHL overexpress bcl-2 is still unclear. Because the bcl-2 protein is widely distributed among different normal lymphohematopoietic cell types and is found in lymphoid, myeloid, and erythroid lineages, techniques for detecting bcl-2 expression at the single cell level are needed to assess the amounts of this protein in normal versus malignant cells. So far, attempts to quantitate bcl-2 expression have generally relied on measurements of the abundance of messenger RNA (mRNA) transcripts, an approach that is not suited for comparative investigations with small cell subpopulations.

We used flow cytometric techniques and a monoclonal antibody (MoAb) that recognizes the human bcl-2 protein to study bcl-2 protein expression in normal human B-cell progenitors and their neoplastic counterparts. B-lineage acute lymphoblastic leukemia (ALL) cells. Our findings indicate that normal B-cell progenitors express low levels of bcl-2 protein, in contrast to ALL cells, which accumulate high levels of the protein despite the absence of detectable rearrangements of the bcl-2 gene. Differences in bcl-2 protein expression correlate with the cells’ ability to survive in vitro and may confer a growth advantage to leukemic lymphoblasts.

MATERIALS AND METHODS

Cells. Bone marrow (BM) samples were taken from eight healthy BM transplantation donors, aged 2 to 23 years (median, 11 years). Normal peripheral blood (PB) samples were collected from five healthy 22- to 37-year-old volunteers. Tonsil samples were from three children undergoing routine tonsillectomies; cell suspensions were prepared with forceps and surgical blades. Leukemic marrow or PB samples (patients 1 and 6, Table 1) were collected at diagnosis from 16 patients, aged 1 to 12 years (median, 6 years), with B-lineage ALL. In all cases, greater than 90% of the blasts expressed CD22, HLA class II antigens, and terminal deoxynucleotidyl transferase (TdT)

From the Department of Hematology-Oncology and the Department of Pathology and Laboratory Medicine, St Jude Children’s Research Hospital, and College of Medicine, University of Tennessee, Memphis; the Clinica Pediatrica Universita, IRCCS Policlinico San Matteo, Pavia, Italy; and the Clinica Pediatrica Universitá di Milano, Ospedale San Gerardo, Monza, Italy.

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Address reprint requests to Dario Campana, MD, PhD, Division of Bone Marrow Transplantation, Department of Hematology-Oncology, St Jude Children’s Research Hospital, 332 N Lauderdale, Box 318, Memphis, TN 38101.

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Mononuclear cells from leukemic samples were cryopreserved within 4 hours of collection and used immediately after thawing. Cells' viability always exceeded 95% by trypan-blue dye exclusion. Normal CD19+ BM cells were obtained by using CD19-immunomagnetic beads (Dynal, Oslo, Norway) and washed three times in phosphate-buffered saline (PBS). After separation on a magnet, cells were detached from the beads using a goat antiserum to mouse Fab (DETACHaBEAD, Dynal). These procedures were performed according to the manufacturer's instructions and yielded cell populations containing 91% to 98% of CD19+ cells, as assessed by flow cytometric analysis (see below).

BM stromal cells were prepared as described in Manabe et al. All culture experiments were performed with AIM-V tissue culture medium (GIBCO, Grand Island, NY; cat. no. 320-2055AJ) without animal sera. Leukemic cells and normal CD19+ cells were resuspended in AIM-V medium at a final concentration of 10^6/mL and placed directly in tissue culture plates or seeded onto marrow stromal cells. All cell cultures were performed in an incubator set at 37°C, 5% CO2, and 90% humidity.

Estimation of bcl-2 protein expression and flow cytometry. The anti-bcl-2 MoAb used in this study was raised by immunizing mice with a synthetic peptide corresponding to amino acids 41 to 54 of the bcl-2 protein. The antibody recognizes a 26-Kd band, identical in size to the bcl-2 protein, in immunoblots under both reducing and nonreducing conditions. Staining with this reagent is confined to the cytoplasm. In the present study, antibody reactivity was analyzed by using suspensions of permeabilized cells. The former were prepared with a Cytospin II Shandon centrifuge (Shandon, Runcorn, UK), air dried, fixed in acetone:methanol 1:1 for 15 minutes at 4°C, and labeled with antibodies and second antibody. In these experiments, anti-

### Table 1. Characteristics of B-Lineage ALL Cases Studied

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/Age</th>
<th>WBC (&gt;10^9/L)</th>
<th>%S*</th>
<th>Immunophenotype†</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/6</td>
<td>72.7</td>
<td>3.0</td>
<td>CD34⁺, CD10⁺</td>
<td>46,XY,t(9;22)(q34;q11)</td>
</tr>
<tr>
<td>2</td>
<td>M/6</td>
<td>56.2</td>
<td>9.2</td>
<td>CD34⁺, CD10⁺, cpl⁺</td>
<td>47,XY,+,del(6)(q15p25),del(11)(q23), del(16)(q22), -1, +del(1)(q21), -q(21), -9, +del(9)(p21;q13), -21, +del<a href="q22.7">21</a></td>
</tr>
<tr>
<td>3</td>
<td>M/1</td>
<td>109.6</td>
<td>8.5</td>
<td>CD34⁺, CD10⁺</td>
<td>46,XY,del(9)(p13), -19, +del(19)(11;19) (q23;p13)</td>
</tr>
<tr>
<td>4</td>
<td>M/10</td>
<td>49.2</td>
<td>3.3</td>
<td>CD34⁺, CD10⁺, cpl⁺</td>
<td>46,XY,del(9)(p13), -19, +del(19)(11;19) (q23;p13)</td>
</tr>
<tr>
<td>5</td>
<td>M/9</td>
<td>372.6</td>
<td>3.8</td>
<td>CD34⁺, CD10⁺</td>
<td>46,XY,t(9;22)(q34;q11)</td>
</tr>
<tr>
<td>6</td>
<td>M/9</td>
<td>870.0</td>
<td>3.5</td>
<td>CD34⁺, CD10⁺</td>
<td>46,XX,t(4;11)(q21;q23)</td>
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<tr>
<td>7</td>
<td>M/6</td>
<td>13.8</td>
<td>2.7</td>
<td>CD34⁺, CD10⁺, cpl⁺</td>
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<td>8.8</td>
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</tr>
<tr>
<td>9</td>
<td>F/5</td>
<td>6.0</td>
<td>2.3</td>
<td>CD34⁺, CD10⁺, cpl⁺</td>
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<tr>
<td>10</td>
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<td>38.7</td>
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<td>CD34⁺, CD10⁺, cpl⁺</td>
<td>46,XY,del(12)(p12)/46,XY,-C,rmar</td>
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<td>11</td>
<td>M/12</td>
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<td>1.7</td>
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<td>7.5</td>
<td>CD34⁺, CD10⁺</td>
<td>45,XX, -12, +del(1)(p32), -del(12) (p7;12)(q11;13)</td>
</tr>
<tr>
<td>13</td>
<td>F/1</td>
<td>186.0</td>
<td>4.8</td>
<td>CD34⁺, CD10⁺</td>
<td>45,XX, -X, -del(12)(p11)</td>
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<td>14</td>
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<td>Not available (DNA index = 1.00)</td>
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<tr>
<td>15</td>
<td>M/4</td>
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<td>8.6</td>
<td>CD34⁺, CD10⁺</td>
<td>47,XY, +21/46,XY,del(9)(p21)</td>
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<tr>
<td>16</td>
<td>F/9</td>
<td>13.7</td>
<td>1.4</td>
<td>CD34⁺, CD10⁺</td>
<td>45,XX, -X, -del(12)(p11)</td>
</tr>
</tbody>
</table>

**Abbreviation**: WBC, white blood cell count at diagnosis.

* Percentage of cells in S phase at diagnosis.

† In all cases, greater than 90% of blasts expressed CD19 and/or CD22, as well as nuclear TdT.

‡ CD34 and cpl expression were not tested in this case.

### Table 2. bcl-2 Protein Expression in Normal BM B Cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>CD19*</th>
<th>CD10⁺</th>
<th>slgM⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.8 (5.2)</td>
<td>5.0 (30.1)</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>3.0 (4.8)</td>
<td>4.1 (9.4)</td>
<td>1.6 (-1)</td>
</tr>
<tr>
<td>3</td>
<td>2.9 (21.6)</td>
<td>2.6 (27.9)</td>
<td>2.3 (20.3)</td>
</tr>
<tr>
<td>4</td>
<td>3.0 (11.6)</td>
<td>2.9 (11.0)</td>
<td>1.8 (2.7)</td>
</tr>
<tr>
<td>5</td>
<td>2.6 (7.4)</td>
<td>2.7 (9.1)</td>
<td>2.0 (6.8)</td>
</tr>
<tr>
<td>6</td>
<td>NT</td>
<td>NT</td>
<td>5.2 (46.9)</td>
</tr>
</tbody>
</table>

**Abbreviation**: NT, not tested.
bcl-2 expression during the different phases of the cell cycle, we labeled BM mononuclear cells with CD10 and goat-anti-mouse IgM PE and permeabilized them as above. The cells were then stained with anti-bcl-2 and goat-antimouse IgG FITC postfixed with 0.5% paraformaldehyde for 10 minutes, washed with DNAse-free RNase (Boeringer Mannheim, Indianapolis, IN; 11.25 Kunitz units), and labeled with 7-actinomycin D (7-AAD; 25 μg/mL; Sigma, St Louis, MO), for at least 30 minutes.21 To investigate Nalm6 cells, we omitted surface staining and labeled the DNA with propidium iodide (PI; 10 μg/mL; Calbiochem, La Jolla, CA). Antibody controls for surface antigens and bcl-2 staining were unreactive MoAbs of IgM (Coulter Immunology, Hialeah, FL) and IgG (Becton Dickinson [BD], San Jose, CA) isotype.

Cells were analyzed with a FACScan flow cytometer with Lysis II software (BD); the CellFit software (BD) was used for cell cycle analysis. The fluorescence intensity of bcl-2 was measured with detectors and amplifiers set on a logarithmic scale. The findings 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 [FCSC], Research Triangle Park, NC), which were run in parallel with the samples in each experiment, as previously described.22,23 Fluorescence intensity achieved with an isotype-matched unreactive antibody was subtracted to that obtained with anti-bcl-2 to calculate the net MESF values. The fluorescence intensities of CD22 (Leu 14; BD) followed by goat-antimouse IgG FITC and of CD19 FITC (Leu 12; BD) were measured with the same technique.

The synthetic peptide GAAPAPGIFSSQPG, analogous to that used for immunization, was synthesized with an Applied Biosystems Peptide Synthesizer 430A (Foster City, CA) with Fmoc HOBt/NMP chemistry.
antihuman Ig light-chain antisera conjugated to FITC (SBA). After two washes in PBSA, the cells were resuspended in 0.5% paraformaldehyde and analyzed with a FACScan and the Lysis II software (BD). In each case, we designed “gates” around the area of the light-scattering properties present in the culture. These numbers were then corrected according to the percentage of cells expressing different antigens present in the culture. These numbers were then corrected according to the percentage of cells expressing different antigens present in the sample studied.

RESULTS

bcl-2 expression during normal B-cell differentiation. The overall expression of bcl-2 within CD19+ cells (including all B-lineage cells) in five normal BM samples was low (MESF, 2,600 to 3,800; median, 3,000; Table 2 and Fig 1). The percentage of CD10+ immature B cells with levels of bcl-2 above control ranged from 9.1% to 30.1%; the intensity of bcl-2 expression within the entire CD10+ cell population ranged from 2,600 to 5,000 (median, 2,900; Table 2 and Fig 1). The expression of bcl-2 within BM slgM+ lymphocytes was heterogeneous (Table 2). In three of five samples, the vast majority of slgM+ cells had low bcl-2 MESF values (1,600, 1,800, and 2,000). By contrast, in the remaining two samples, staining of slgM+ cells for bcl-2 yielded a bimodal histogram corresponding to two separate cell populations. The MESF in the cells with low bcl-2 expression was 1,600 in one sample and 2,000 in the other; in the cells with high bcl-2 expression, the values were 23,000 and 32,000, respectively (Table 2). The expression of bcl-2 in the latter cells was similar to that seen in B lymphocytes from five PB samples, in which greater than 90% of slgM+ cells expressed bcl-2 with overall MESF values of 25,000 to 36,000 (median, 30,000). Similar observations were made when the antihuman IgM reagent was substituted with a mixture of anti-κ and anti-λ human light-chain antibodies (data not shown).

When studied by fluorescence microscopy, 42% to 65% of TdT+ progenitor cells had detectable staining with anti-bcl-2. These cells were heterogeneous in size, with some appearing as small lymphoid cells and others being large blasts with nucleoli.

Expression of bcl-2 protein was also studied in three tonsil samples, in which 16.4%, 19.1%, and 44.1% of cells were CD10+; 89.5%, 72.9%, and 84.1% of these cells had weak or absent bcl-2 expression, with overall MESF values of 5,000, 5,800, and 3,500, similar to that observed in CD10+ cells in BM (Table 2 and Fig 1). Tonsil CD10+ cells include reactive germinal center cells and these data are in line with the observation that most of these cells have undetectable levels of bcl-2 on immunohistologic examination.

Increased bcl-2 expression in B-lineage ALL and immature B-cell lines. Sixteen samples of B-lineage ALL and five continuously growing B-lineage cell lines were also studied. In all leukemic cases, greater than 80% of cells showed bcl-2 staining higher than the highest intensity observed with the isotype-matched negative control antibody; the MESF ranged from 14,000 to 68,000 (Table 3). Cell staining was completely abolished when the anti-bcl-2 antibody was preincubated with the peptide used for immunization. Thus, leukemic cells expressed strikingly higher levels of bcl-2 than did their normal CD10+ counterparts (mean MESF ± SD of 33,560 ± 14,570 vs 3,460 ± 1,050; P < .001 by t-test). However, the intensity of bcl-2 in cell lines (79,400 ± 20,330) was even higher than the value for the ALL cases (P < .001; Table 3).

Expression of bcl-2 by leukemic cells did not correlate with the patients’ clinical features at diagnosis. The initial white blood cell (WBC) count in this series ranged from 6.0 to 870 × 10⁹/L, without any linear relationship to bcl-2 expression (r = −.164; Tables 1 and 3).

We also attempted to relate the intensity of bcl-2 expression to the leukemic cell karyotype, which was available for 12 cases. None of the cases had metaphases with the t(14;18), the translocation found in the majority of cases of follicular B-cell NHL in adults, or abnormalities involving IgHq21 (Table 1).

| Table 3. Quantitation of bcl-2, CD22, and CD19 Expression in B-Lineage ALL |
|-----------------|-----------------|-----------------|
| Sample          | bcl-2           | CD22            | CD19            |
| Patients        |                 |                 |                 |
| 1               | 14              | 26              | 27              |
| 2               | 16              | 14              | 13              |
| 3               | 18              | 15              | 24              |
| 4               | 20              | 25              | 16              |
| 5               | 22              | 9               | NT              |
| 6               | 30              | 9               | 14              |
| 7               | 31              | 20              | 16              |
| 8               | 31              | 10              | NT              |
| 9               | 32              | 15              | 17              |
| 10              | 34              | 15              | 8               |
| 11              | 36              | 15              | NT              |
| 12              | 40              | 19              | 14              |
| 13              | 45              | 13              | 15              |
| 14              | 49              | 13              | NT              |
| 15              | 51              | 16              | 28              |
| 16              | 68              | 14              | 16              |
| Cell lines      |                 |                 |                 |
| 380             | 49              | 14              | NT              |
| Nalm6           | 70              | 19              | NT              |
| SUPB15          | 85              | 35              | NT              |
| MR87            | 93              | 32              | NT              |
| REH             | 100             | 30              | 30              |

Values are MESF × 10². Abbreviation: NT, not tested.

| Table 4. Survival of Normal BM B Cells on Allogeneic Stromal Feeder Layers |
|-----------------|-----------------|-----------------|-----------------|
| Experiment No.  | CD19+ Cells     | Within CD19+ Cells* |
|                 | Recovered (%)   | CD34+, slgκ     | CD34+, slgκ−   |
| 1               | 30.5±           | 2.8             | 30.1           | 73.1 |
| 2               | 14.2            | 2.7             | 11.2           | 35.0 |
| 3               | 30.0            | 23.7            | 28.8           | 39.2 |

* Different subsets of BM B cells were defined by triple-color flow cytometric analysis (see text).
† CD19+ cells were purified as described in Materials and Methods before seeding onto BM stromal layers. The percentage of CD19+ cells at the beginning of the culture in the three experiments was 92, 98, and 91, respectively.
‡ Values represent the percentage of cells expressing the indicated phenotype recovered after 7 days of culture on stroma. Cell numbers were calculated by flow cytometry at day 0 and day 7 (see text).
Leukemic lymphoblasts survive longer than normal B-cell progenitors. In a previous study, we demonstrated that allogeneic BM stromal feeder layers prevent apoptosis and support optimal survival of leukemic lymphoblasts in vitro. In the present study, we compared the ability of these feeder layers to maintain normal and leukemic B-cell progenitors in vitro. Twelve of the 16 cases of B-lineage ALL included in this study were tested. Cell numbers were investigated at the beginning and after 7 days of culture and the percentage of cells recovered was 68% to 178% (median, 95.5%) of those originally seeded. By contrast, the number of normal CD19+ cells considerably decreased under identical culture conditions and less than one-third were recovered after 7 days in three samples studied (Table 4). Cell loss was severe within the rapidly dividing CD19+, slg- immature B-cell compartment. Triple-color analysis by flow cytometry showed that the most immature CD19+ cells, identified by the expression of CD34, were also the cells disappearing most rapidly (Table 4).

bcl-2 expression correlates with survival requirements of leukemic cells. A further heterogeneity in cells life span associated with different expression of bcl-2 protein was found within the B-lineage ALL cases. We previously observed that in some cases a substantial proportion of cells can be recovered even after prolonged periods of culture without stromal cells. Phenotypic or karyotypic markers that would distinguish cases requiring stromal support from those capable of surviving in serum-free medium have not been identified. In 6 of the 12 cases included in the present study (nos. 9, 10, 12, 13, 15, and 16), no signs of apoptosis were seen after 72 hours of culture in the absence of stroma (Fig 2). The mean ± SD MESF of bcl-2 in these cases was 45,000 ± 13,270. In the other six cases (nos. 1 through 4, 6, and 7), signs of massive apoptosis were evident within 3 days of culture in serum-free medium (Fig 2). The mean ± SD MESF of bcl-2 in these cases was significantly lower than in the previous group (21,500 ± 7,260; P = .003). By contrast, the expression of...
CD22 and CD19 in both groups was similar (15,330 ± 2,070 vs 18,170 ± 6,680 for CD22; 16,330 ± 6,530 vs 18,330 ± 5,750 for CD19). In all cases in which signs of rapid apoptosis were observed, less than 0.5% of the cells were viable after 7 days of culture. By contrast, in the cases with higher bel-2 protein expression, a proportion of nonapoptotic CD19+ cells (20% to 95% of the original population) persisted after 7 days of culture. However, the percentage of cells recovered (95% in patient 9; 90% in patient 10; 67% in patient 11; 62% in patient 12; 57% in patient 13) did not have a linear relation to the intensity of bel-2 expression, suggesting that other factors had influenced the expansion of ALL cells.

Constant bel-2 expression during the cell cycle. The percentage of cells in S phase at diagnosis ranged from 1.4% to 22.6% among the 16 cases of ALL; these values did not strongly correlate with bel-2 expression (r = .146; Tables I and 3). To investigate whether the expression of bel-2 changes as cells progress through the cell cycle, we simultaneously labeled a pre-B-leukemia cell line, Nalm 6, for bel-2 and DNA content. Normal B-cell progenitors from two marrow samples were also labeled by this method; the immature B cells were recognized by CD10 labeling, with triple-color immunofluorescence used to study the samples. The expression of bel-2 remained constant in both leukemic and normal progenitor cells throughout the different phases of the cell cycle (Fig 3).

DISCUSSION

In this study of the expression of the bel-2 protein among normal and leukemic human B cells, we showed that bel-2 expression is low in immature B-cell precursors, which are, in this respect, similar to reactive germinal center cells. Moreover, during B-cell differentiation, levels of bel-2 protein are at their lowest in B-lineage cells that proliferate in central and peripheral lymphoid organs. It is unlikely that the fluctuations of bel-2 levels we observed are related to cell cycle because continuously growing cell lines had the highest levels of bel-2 protein and cells in S, G2, or M phase did not have lower levels of bel-2 expression than cells in G0 or G1. In addition, bel-2 expression in B-lineage ALL did not correlate with the proportion of cells in S phase.

Low expression of bel-2 in germinal center cells could be instrumental in allowing selection mechanisms based on affinity for antigen to operate through apoptosis. Immature B-cell progenitors are also likely to undergo apoptosis during normal development because rearrangements of Ig genes may be unsuccessful in a large proportion of cells; it has been postulated that less than 10% of these progenitors mature to slg+ B lymphocytes. Low levels of bel-2 expression at this stage of differentiation may be advantageous, facilitating the swift demise of ineffective progenitor cells. The rapid disappearance of these cells was observed when purified normal BM B cells were cultured under conditions that can support the expansion of leukemic B cells for several months. Only a fraction of CD19+, slg- cells could be recovered after 7 days of culture despite the presence of stromal feeder layers. It is unlikely that the disappearance of these rapidly proliferating progenitor cells was entirely due to cell maturation because the numbers of slg+ lymphocytes in our cultures did not increase. It is possible that improved culture conditions and the addition of cytokines may allow greater numbers of normal immature B cells to survive and differentiate in culture (A. Manabe and D. Campana, unpublished observations). However, in the culture system used in this study, they appear to be more vulnerable than their neoplastic counterparts.

High levels of bel-2 protein in leukemic cells may lead to a prolonged lifespan, and lend a growth advantage to these cells despite their lower proliferation rate. BM-derived stromal cells support long-term culture of most ALL samples. In the absence of stromal feeder layers, cultured cells rapidly die by apoptosis in most cases. In this respect, these cases of ALL resemble factor-dependent hematopoietic cell lines cultured under suboptimal conditions. However, in a proportion of ALL cases, delayed onset of apoptosis and prolonged survival of cells in vitro is seen in the absence of stromal feeder layers. In this study, we observed that these latter cases had a higher bel-2 expression. These results are consistent with experiments showing that transfection of bel-2 in cytokine-dependent cell lines retards the initiation of cell death in unfavorable culture conditions.

Our findings support the concept that, during normal B-cell development, bel-2 protein levels are low at stages of differentiation when apoptosis may be physiologically triggered (eg, in cells that have failed to rearrange Ig genes, and in antigen-responding clones with low affinity for the antigen). Overexpression of bel-2 in cells susceptible to apoptosis is likely to favor the survival of immunologically inadequate cells. A consequence of high bel-2 expression in B-cell progenitors would be the prolonged survival of cells with nonproductive Ig heavy- or light-chain gene rearrangement, a characteristic of virtually all cases of B-lineage ALL. In NHL, the t(14;18) abnormality leads to overexpression of bel-2. We could not find karyotypic evidence of bel-2 gene rearrangement in the B-lineage ALL cases. Structural abnormalities of chromosome 18q21 are extremely rare in childhood ALL, and only two children with NHL and the t(14;18) have been reported in the literature. The mechanism for increased expression of bel-2 in other cases of ALL remains to be identified.

In conclusion, the results of this study suggest that regulation of bel-2 expression plays an important role in the development of human B cells and that abnormal gene expression may contribute to leukemogenesis by prolonging the life span of progenitor cells unable to further differentiate. In addition, overexpression of bel-2 may facilitate the expansion of the leukemic clones outside the BM microenvironment. This study cannot address whether the expression of bel-2 correlates with treatment outcome in ALL, as all patients remain in continuous remission with a relatively short follow-up (3 to 46 months; median, 9 months). Further studies may disclose the value of bel-2 expression as a prognostic indicator and its association with clinical features of ALL, such as occurrence of extramedullary relapse and resistance of leukemic cells to drugs.

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