The **bcl-2** gene becomes transcriptionally deregulated in the majority of low-grade non-Hodgkin lymphomas as a result of t(14;18) translocations that place the bcl-2 gene at 18q21 into juxtaposition with the Ig heavy-chain locus at 14q32. This chromosomal translocation or similar bcl-2 gene rearrangements involving the Ig light-chain genes have been reported to occur in several cases of B-cell chronic lymphocytic leukemia (B-CLL). We analyzed the structure, methylation, and expression of the bcl-2 gene in 20 cases of B-CLL or closely related variants of this lymphoproliferative disorder, including at least 16 typical examples of CD5+ B-CLL. None of the 20 specimens had evidence of bcl-2 gene rearrangements, based on Southern blot analysis using three different bcl-2 probes. However, immunoblot analysis using antibodies specific for the Bcl-2 protein showed that 14 of 20 cases (70%) contained levels of p26-Bcl-2 that were equal to or greater than those found in a t(14;18)-bearing lymphoma cell line. Furthermore, in 19 of 20 cases (95%), the Bcl-2 protein was present at levels that were 1.7- to 25-fold higher than in normal peripheral blood lymphocytes. These differences in the relative levels of Bcl-2 protein among cases of B-CLL appeared to be functionally significant, in that a preliminary analysis of 3 representative cases showed that CLL cells with higher levels of Bcl-2 protein survived longer in culture and were delayed in their onset of DNA degradation relative to CLL cells with lower Bcl-2 protein levels. Evaluation of the methylation status of the bcl-2 gene using the isoschizomers Msp I and Hpa II, and a probe corresponding to the first major exon of the gene showed complete demethylation of both copies of the bcl-2 gene in a region corresponding to a 2.4-kb Msp I fragment in all 20 cases of B-CLL. In contrast, analysis of 6 of 6 B-cell lines that harbor a t(14;18) was consistent with hypomethylation of only one of the two bcl-2 alleles. Neither copy of the bcl-2 gene was demethylated in this region in 5 of 5 lymphoid cell lines that lack this translocation. However, hypomethylation of the bcl-2 gene did not necessarily correlate with the relative levels of Bcl-2 protein present in the B-CLL cells, suggesting that additional mechanisms for regulating bcl-2 expression are involved. Taken together, the data indicate that high levels of bcl-2 expression occur frequently in B-CLL, and suggest that trans-regulatory mechanisms are involved in this lymphoproliferative disorder, as opposed to the cis-regulatory defect commonly found in t(14;18)-containing lymphomas.

© 1993 by The American Society of Hematology.
ently incurable, although recent advances in autologous bone marrow purging and transplantation may change the ultimate outcome for at least some patients with low-grade lymphomas. Part of the explanation for the incurability of follicular lymphoma and B-CLL may be related to the low mitotic rate of the cells, given that the majority of chemotherapeutic drugs currently used in the treatment of cancer are relatively specific for rapidly proliferating cells. However, another potential contributor to the poor outcome for patients with follicular lymphoma may be the bcl-2 gene itself, whose protein product can increase the relative resistance of cells to killing by essentially all chemotherapeutic drugs and by γ-irradiation. These similarities between B-CLL and the low-grade NHLs prompted us to more closely examine the status of the bcl-2 gene in leukemic blood specimens derived from 20 patients affected with B-CLL or its variants.

MATERIALS AND METHODS

Patient materials. All clinical specimens represented peripheral blood samples derived from patients from either Milan, Italy (cases no. 1 through 18) or Philadelphia, PA (cases no. 19 and 20). Blood cells were centrifuged on ficoll to obtain the mononuclear cell fraction, as described previously. These cells were either used when fresh, or frozen while viable in Iscove's modified Eagle's medium (IMEM) containing 20% (vol/vol) fetal calf serum and 10% (vol/vol) dimethylsulfoxide, and stored in liquid nitrogen. After thawing, dead cells were removed by centrifugation on ficoll. None of the patients had begun treatment at the time of sample acquisition. Immunophenotyping for CD5 expression was accomplished as described previously.

Cell lines. Cell lines used for these studies included the t(14;18)-containing NHL lines RS11846, WSU-NHL, DoHH2, SU-DHL-6, and Karps 422; the t(14;18)-containing pre-B-cell ALL line 380; the t(1;19)-containing pre-B-cell ALL line 697; the t(6;14)-containing Burkitt line Daudi; the T-cell ALL line Jurkat; and the lymphoblastoid B-cell lines AKATA, CHEP, and BJAB. All cells were maintained in culture using RPMI medium containing 10% fetal calf serum and harvested while in log-phase of growth.

Immunoblot assay. Analysis of relative levels of Bcl-2 and other proteins in B-CLL cells was accomplished by immunoblot assay exactly as described previously.20 Protein-containing lysates were prepared from fresh or frozen cells for all 20 cases. Samples were normalized for total protein content and 100-μg aliquots analyzed one to three times (depending on the amount of protein obtained). Blots were cut into sections and incubated with various antibodies stained with Ponceau-S (Sigma, St Louis, MO) to verify loading of approximately equal amounts of total protein. Antibodies used for these studies included two different rabbit antisera that were raised against synthetic peptides corresponding to amino acids 41 through 54 or 61 through 76 of the human Bcl-2 protein, a rabbit antiserum specific for the 50-Kd inner mitochondrial protein F1-β-ATPase, and a monoclonal antibody 19F4 that recognizes the 36-Kd subunit of DNA polymerase delta (PCNA; purchased from Boehringer Mannheim, Inc, Indianapolis, IN). Antibody binding to filters was detected by use of 125I-protein A and exposure to x-ray film. The relative intensity of bands on the resulting autoradiograms was quantified by scanning densitometry (Ultroscan III; LKB Instruments, Inc, Bromma, Sweden) where the area under the tracing was calculated. Comparable results were obtained with both of the anti-Bcl-2 antibody reagents; therefore, the results were combined.

Southern blotting. Genomic DNA was isolated from B-CLL cells or from various cell lines, and 10-μg aliquots were analyzed by Southern blotting, as described previously.3,30 The DNA hybridization probes included a 3.85-kb Xho I to HindIII fragment from p18-21H (5′-bcl-2 probe), a 4.4-kb HindIII fragment from p18-4 (3′-bcl-2 probe; MBR), a 4.5-kb EcoRI fragment from pFL2-(bcl-2 MCR) probe, and a 2.8-kb Mbo I fragment from pKB-Mbol 2.8 (human mitochondrial DNA probe).3,23,24

Cell viability and DNA content analysis. Frozen B-CLL specimens were thawed, and dead cells were removed by centrifugation on ficoll so that the starting cell number was greater than 99% viable based on trypan blue dye exclusion. One million cells per milliliter were then cultured at 37°C and 5% CO2 in IMEM containing 20% (vol/vol) heat-inactivated fetal calf serum, 1 mmol/L glutamine, 50 U/ml penicillin, and 100 μg/ml streptomycin. Cells were recovered from cultures at various times, and either fixed for 30 minutes on ice in Dulbecco's phosphate-buffered saline (PBS) or resuspended in PBS containing 0.2% (final) trypan blue and immediately counted using a hemocytometer to determine the percentage of viable cells. A minimum of 200 cells were counted per sample, and the data were reported as the mean ± standard deviation for three determinations.

The relative DNA content of fixed cells was estimated by flow cytometric analysis. Cells were placed overnight at 4°C in PBS (pH 7.4) containing 50 μg/ml propidium iodide, 0.1% Triton-X100, 0.1 mmol/L EDTA, and 50 μg/ml RNase-A and then analyzed using a Becton Dickinson FACScan instrument (Becton Dickinson, Mountain View, CA), essentially as described previously.21 Data were displayed as histograms, representing relative fluorescence intensity (abscissa) versus relative cell number (ordinate). A lower limit threshold for fluorescence was set on the cells with G0/G1 DNA content and the percentage of propidium iodide-stained cells having fluorescence less than this value was determined.

RESULTS

High relative levels of p26-Bcl-2 protein occur frequently in B-CLL. Table 1 presents some of the characteristics of the leukemic cell specimens evaluated with regards to bcl-2 gene expression. Of the 20 cases, 17 were diagnosed as BCLL based on morphology, lymphocyte count, bone marrow examination, and clinical presentation. One was a hairy-cell variant of CLL (HCV). One case was morphologically consistent with prolymphocytic leukemia (PLL), and another represented a patient who presented with concomitant well-differentiated, small noncleaved cell lymphoma (NHL). Surface marker data were available for 19 of the 20 specimens, showing that at least 16 of the cases were CD5+. Included among the CD5+ specimens were the HCV and PLL cases (Table 1).

Figure 1 shows an example of some immunoblot data for 10 of the 20 B-CLL cases, and Table 1 summarizes the data for all 20 specimens. All data were compared with RS11846, a B-CLL lymphoma line that harbors a t(14;18), and with peripheral blood lymphocytes (PBLs) from normal volunteers. Note that normal PBLs (which are composed of mostly T cells) contained relative levels of p26-Bcl-2 protein that were only about 20% of those found in the lymphoma cell line. With the exception of one sample (case no. 3), mononuclear cells recovered from B-CLL samples contained higher levels of p26-Bcl-2 protein than normal PBLs. In 14 of 20 cases, the levels of Bcl-2 protein in B-CLL cells
The percentage of cells exhibiting immunostaining with an antibody specific for CD5 was determined by flow-cytometric immunofluorescence analysis of 3 human lymphoblastoid cell lines (AKATA, BJAB, and CHEP) that lack a t(14;18), contain relatively low levels of Bcl-2 protein, and that are very similar to the t(14;18)-positive lymphoma lines with regards to their stage of B-cell differentiation (as defined by surface markers and Ig gene rearrangements) showed complete methylation in the region of bcl-2 corresponding to this 2.4-kb MspI restriction fragment. Methylation of this same region of bcl-2 was also found in all other lymphoid cell lines examined that lack a t(14;18) and that have relatively low levels of Bcl-2 protein, including the pre-B–cell ALL line 697 and the T-lymphoblastoid cell line Daudi.

Hypomethylation of the 5′-end of the bcl-2 gene in cell lines containing a t(14;18). Previously, we examined the methylation status of the bcl-2 gene in two B-cell lines that contain a t(14;18), and found that one of the two copies of the bcl-2 gene in these cells was hypomethylated in the region corresponding to the first major exon of the gene. This correlation between methylation and bcl-2 gene rearrangement is most easily appreciated by examination of a 2.4-kb XhoI to HindIII bcl-2 genomic probe. As shown in Fig 2, approximately half of the DNA derived from the t(14;18)-containing cell lines 380, SU-DHL-6, and RS11846 was cleaved by the methylation-sensitive enzyme HpaII, thus producing a 2.4-kb band in these Southern blot assays. Essentially identical results were obtained for the t(14;18)-containing cell lines WSU-NHL, Karpass 422, and DoHH2 (not shown). Presumably, the hypomethylated DNA corresponds to the rearranged copy of the bcl-2 gene, because analysis of 3 human lymphoblastoid cell lines (AKATA, BJAB, and CHEP) that lack a t(14;18), contain relatively low levels of Bcl-2 protein, and that are very similar to the t(14;18)-positive lymphoma lines with regards to their stage of B-cell differentiation (as defined by surface markers and Ig gene rearrangements) showed complete methylation in the region of bcl-2 corresponding to this 2.4-kb MspI restriction fragment. Methylation of this same region of bcl-2 was also found in all other lymphoid cell lines examined that lack a t(14;18) and that have relatively low levels of Bcl-2 protein, including the pre-B–cell ALL line 697 and the T-lymphoblastoid cell line Daudi.

were as high or higher than those found in the t(14;18)-containing lymphoma cell line. Among the 6 cases that contained relatively less Bcl-2 protein, 4 were CD5+ B-CLL, 1 was a CD5+ HCV, and for 1 case no immunophenotypic data were available. Incubation of blots with an antiserum specific for the mitochondrial inner membrane protein F1β-ATPase verified the presence of intact protein for all samples.

Some blots were also incubated with a monoclonal antibody specific for PCNA, because a previous report suggested that this proliferation-associated protein was a marker of worse prognosis for at least some patients with B-CLL. Only 3 cases of B-CLL contained detectable PCNA (Table 1). These data were normalized relative to the levels of PCNA found in Daudi, a B-cell lymphoma cell line that contains a t(8;14) involving c-myc and that has a high proliferative rate. Among the cases that contained PCNA, 2 had relatively high levels of Bcl-2 protein (cases no. 4 and 11) and one had lower levels of this oncoprotein (case no. 7). All were CD5+.

---

**Table 1. Patient Samples and Immunoblot Results**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>CDS (%</th>
<th>BCL-2* (mean ± SD)</th>
<th>No. of Experiments</th>
<th>PCNA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CLL</td>
<td>80</td>
<td>2.14 ± 0.78</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>HCV</td>
<td>0</td>
<td>0.46 ± 0.27</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>CLL</td>
<td>71</td>
<td>0.05</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>CLL</td>
<td>83</td>
<td>2.53 ± 1.58</td>
<td>3</td>
<td>0.45</td>
</tr>
<tr>
<td>5</td>
<td>CLL</td>
<td>66</td>
<td>0.96 ± 0.17</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>CLL</td>
<td>95</td>
<td>0.33</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>CLL</td>
<td>53</td>
<td>0.41 ± 0.11</td>
<td>3</td>
<td>0.03</td>
</tr>
<tr>
<td>8</td>
<td>CLL</td>
<td>79</td>
<td>0.51 ± 0.06</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>CLL</td>
<td>90</td>
<td>1.17 ± 0.59</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>NHL</td>
<td>90</td>
<td>1.60 ± 0.68</td>
<td>3</td>
<td>0.69</td>
</tr>
<tr>
<td>11</td>
<td>CL2</td>
<td>54</td>
<td>1.79 ± 0.88</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>CL2</td>
<td>ND</td>
<td>1.73 ± 0.12</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>PLL</td>
<td>0</td>
<td>1.39 ± 0.29</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>CL2</td>
<td>50</td>
<td>1.39 ± 0.21</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>CL2</td>
<td>69</td>
<td>5.02</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>CL2</td>
<td>83</td>
<td>1.91 ± 0.59</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>CL2</td>
<td>89</td>
<td>1.95 ± 0.32</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>CL2</td>
<td>99</td>
<td>1.11 ± 0.13</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>CL2</td>
<td>98</td>
<td>0.78 ± 0.69</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Peripheral blood lymphocytes 0.20 ± 0.12 7 0

PBLs from 7 normal volunteers or from 20 patients affected with B-CLL or its variants were analyzed by immunoblotting for their relative levels of Bcl-2 and PCNA proteins. The diagnosis for leukemia patients was established by consideration of morphologic and immunophenotypic data, and determined to be consistent with CLL, HCV, NHL, or PLL. The percentage of cells exhibiting immunostaining with an antibody specific for CD5 was determined by flow-cytometric immunofluorescence assay.

Abbreviation: ND, not done.

* Immunoblot data derived from the analysis of 100-μg aliquots of protein lysates were quantified by scanning densitometry. The data were normalized relative to the t(14;18)-containing B-lymphoma line RS11846, which was included on every blot as an internal control. Each B-CLL sample was analyzed two or three times in separate experiments (number in parentheses) and the data reported as the mean of two determinations or as the mean ± standard deviation for three assays. PBLs from 7 normal volunteers were also analyzed.

† Immunoblot data for PCNA protein levels were normalized relative to the results obtained with the Burkitt lymphoma line Daudi, which was included on every blot as an internal control.

---

![Image of immunoblot analysis](https://example.com/immunoblot.png)

**Fig 1.** Immunoblot analysis of Bcl-2, PCNA, and F1β-ATPase proteins in B-CLL cells. Ficoll-purified cells were obtained from the peripheral blood specimens of 10 patients affected with B-CLL or from the PBLs of a normal volunteer. Triton-X100 extracts were prepared as described previously, normalized for total protein content (100 μg per lane), and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gels), followed by transfer to nitrocellulose. Blots were cut into sections and incubated with antibodies specific for either p26–Bcl-2, p36–PCNA, or p50–F1β-ATPase. As controls, 100-μg aliquots of protein from the cell lines RS11846 and Daudi were also analyzed.

---

From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
BCL-2 EXPRESSION IN B-CLL

Fig 2. DNA methylation analysis of bcl-2 in cell lines that either lack or contain a t(14;18) translocation. DNA was isolated from various cell lines and 12 μg was digested to completion with either Msp I (M) or Hpa II (H) before electrophoresis in 0.8% agarose gels. After transfer to nylon membranes, blots were hybridized with a 32P-labeled 3.85-kb Xho I to HindIII fragment from the p18-21H probe, representing a genomic clone of a region of the bcl-2 gene corresponding to portions of the first major exon and extending into the first 2.6 kb of the large intron in this gene. Markers represent HindIII-digested λ DNA (in kilobase pairs). For the lower panel, the bcl-2 probe was eluted and the blot was rehybridized with a 32P-labeled mitochondrial DNA probe. Only the region of the blot corresponding to the 1.2-kb mitochondrial Msp I fragment is shown.

Fig 3. DNA methylation analysis of the bcl-2 gene in B-CLL cells. DNA was isolated from peripheral blood mononuclear cells of 4 patients with B-CLL or from the B-lymphoblastoid line AKATA. After digestion of 10 μg with Msp I (M) or Hpa II (H), DNAs were size-fractionated in 0.8% agarose gels, transferred to nylon membranes, and hybridized with a 32P-labeled 3.85-kb Xho I to HindIII fragment from p18-21H. After exposure to x-ray film, the bcl-2 probe was eluted and the blot was rehybridized with a mitochondrial DNA probe (lower panel). Data are representative of all 20 cases of B-CLL.

Hypomethylation of the 5'-end of the bcl-2 gene occurs frequently in B-CLL and involves both copies of the gene. Sufficient DNA was obtained from all 20 B-CLL specimens for analysis of bcl-2 gene methylation. Blots containing DNA cleaved with either Msp I or Hpa II were sequentially hybridized with probes corresponding to various regions of bcl-2 as well as with a mitochondrial DNA probe to verify complete digestion of DNA with Hpa II because mitochondrial DNA is not subject to methylation.

Analysis of DNA methylation in the 3'-end of the bcl-2 gene was accomplished using a 4.4-kb HindIII genomic probe that hybridizes near the MBR in the 3'-untranslated portion of the gene. Hybridization of Msp I-digested DNA with this probe showed bands of 4.3, 3.6, 2.1, and 1.3 kb in all samples (not shown). In only 3 cases were any of these bands present in Hpa II-digested DNA, indicating that the 3'-end of the bcl-2 was extensively methylated. The exceptions were cases no. 4 and 5, in which the 4.3-kb band was

For personal use only.on September 24, 2017. For personal use only.
were detected.

determine the extent of DNA methylation using gene near the MCR. The extent of DNA methylation was reported as heavy methylation; N, no methylation; G, germline; R, rearranged; ND, only the germline restriction fragments (G), and no rearranged bands (R) and

Table 2. Southern Blot Analysis of bcl-2 Gene Methylation and Rearrangement in B-CLL

<table>
<thead>
<tr>
<th>Case No.</th>
<th>5'-Probe</th>
<th>MBR</th>
<th>MCR</th>
<th>5'-Probe</th>
<th>MBR</th>
<th>MCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N</td>
<td>H</td>
<td>M</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>3</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>4</td>
<td>N</td>
<td>M</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>5</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>6</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>8</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>9</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>10</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>11</td>
<td>N</td>
<td>L</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>12</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>13</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>14</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>15</td>
<td>N</td>
<td>M</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>16</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>17</td>
<td>N</td>
<td>H</td>
<td>M</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>18</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>19</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>20</td>
<td>N</td>
<td>H</td>
<td>H</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
</tbody>
</table>

Abbreviations: L, light methylation; M, moderate methylation; H, heavy methylation; N, no methylation; G, germline; R, rearranged; ND, not done.

* Genomic DNA was analyzed using the enzymes Msp I and Hpa II to determine the extent of DNA methylation using bcl-2 probes corresponding to the 5'-end of the gene, the 3'-end near the MBR, and 3' of the gene near the MCR. The extent of DNA methylation was reported as heavy (H), moderate (M), light (L), or none (N) (see text for details).

† The structure of the bcl-2 gene was analyzed by Southern blot analysis of DNA isolated from B-CLL cells, using the enzymes HindIII, BamHI, and EcoRI and using three different bcl-2 probes. All samples contained only the germline restriction fragments (G), and no rearranged bands (R) were detected.

hypomethylated, and case no. 12, in which both the 4.3- and 2.1-kb bands were present in Hpa II-digested DNA (Table 2). Similarly, analysis of DNA methylation in a region 3' of the bcl-2 transcriptional unit using a 4.5-kb EcoRI genomic probe that hybridizes near the bcl-2 MCR showed heavy methylation in 18 of 20 B-CLL samples (Table 2). In these cases, Hpa II-digested DNA contained a band of ≥25 kb, in contrast to Msp I bands of 2.9, 3.0, and 3.1 kb (not shown). In 2 cases, an Hpa II band of 3.2 kb was present, suggesting less extensive methylation.

Absence of bcl-2 gene rearrangements in B-CLL samples. In 19 of 20 cases of B-CLL, sufficient DNA was available for analysis of bcl-2 gene structure using the restriction enzymes HindIII, BamHI, and EcoRI, and the three bcl-2 probes described above. Previous studies have shown that Southern blot analysis using these three enzymes and probes detects 90% of t(14;18) translocations. As summarized in Table 2, no evidence of bcl-2 gene rearrangements was obtained for any of the 19 B-CLL cases examined. In the 1 case (patient no. 6) in which insufficient DNA was obtained for digestion with HindIII, BamHI, and EcoRI, no rearrangements were detected in the Msp I-digested DNA, suggesting that this case also lacked bcl-2 gene rearrangements (not shown).

Correlation of Bcl-2 protein levels with in vitro survival of B-CLL cells. Overexpression of the bcl-2 gene has been reported to prolong the in vitro survival of B lymphocytes by delaying the onset of programmed cell death or apoptosis. As a first attempt to evaluate the functional significance of the high levels of Bcl-2 protein found in many cases of B-CLL, we compared the in vitro survival of 3 CLL specimens for which additional cells were still available: samples no. 3, 10, and 16 (see Table 1). Samples no. 3 and 16 contained the lowest and the highest relative levels of Bcl-2 protein, respectively, among the cases of B-CLL analyzed here. Case no. 10 contained an intermediate amount of Bcl-2 protein. As shown in Fig 4, the B-CLL cells with low levels of Bcl-2 protein (case no. 3) died more quickly in culture than cells that contained relatively high levels of this oncoprotein (cases no. 10 and 16). These cells with low Bcl-2 also became shrunken and developed a morphologic appearance typical of apoptotic cells (not shown).

One of the hallmarks of apoptotic cell death is the degradation of nuclear DNA due to the activation of intracellular endonucleases. We therefore estimated the DNA content of these same B-CLL cell specimens at various times after initiation of in vitro cultures. Figure 5 shows a comparison of the results for B-CLL specimens no. 16 (high Bcl-2 protein) and 3 (low Bcl-2 protein). Initially, all cells in these cultures exhibited propidium-iodide (PI) staining typical of G2/G1-phase cells. However, within 6 hours, a population of cells with reduced PI-staining began to appear in the cultures of B-CLL cells having low levels of Bcl-2 protein but
not in the B-CLL specimen having high levels of Bel-2 protein. By 72 hours, the majority of cells recovered from cultures of specimen no. 3 contained reduced levels of PI-stainable DNA consistent with apoptosis, whereas most of the specimen no. 16 cells retained their starting G$_0$/G$_1$-phase DNA content. Those cells with reduced DNA content also exhibited lower forward light scattering values, consistent with the cell shrinkage that characterizes apoptotic cells (not shown).

**DISCUSSION**

The biologic and clinical similarities in B-CLL and follicular NHL prompted us to more closely examine the status of bcl-2 gene structure, methylation, and expression in B-CLL. In contrast to most follicular lymphomas, no evidence of bcl-2 gene rearrangements was found in any of the 20 patient samples examined here. Previous studies of bcl-2 gene structure in B-CLL have shown that fusion of bcl-2 with Ig gene loci can occur in a small percentage of cases. Adachi et al.\textsuperscript{6} for example, detected rearrangements in the 5'-end of bcl-2 in 3 of 32 cases of B-CLL examined. In each case, molecular cloning confirmed fusion of bcl-2 with either the Ig k- or \(\lambda\)-gene loci. Raghoeber et al\textsuperscript{7} studied 44 cases of B-CLL and found 3 with evidence of bcl-2 gene rearrangement by Southern blotting. One of these represented a t(14;18) translocation involving the MBR in a CD5$^+$ B-
CLL, whereas the other 2 cases had rearrangements in the 5’-end of bel-2 and were CD5+. In contrast, Rechavi et al.40 detected no bel-2 gene rearrangements in their analysis of 38 patients with B-CLL. More recently, Schena et al.31 and Mariano et al.32 studied 9 and 20 cases, respectively, of B-CLL and also found no evidence of bel-2 gene rearrangements in their patient samples. When combined with the data reported here, therefore, the incidence of bel-2 gene rearrangements in CLL appears to be quite low (6 of 163 cases reported [4%]).

Despite the absence of detectable bel-2 gene rearrangements, in 70% of 20 cases examined here the relative levels of p26-Bcl-2 protein in B-CLL cells were equivalent to or greater than those found in a reference cell line that contains a t(14;18). Similarly, levels of bel-2 mRNA were recently reported to be 4 to 31 times higher than those found in normal PBLs in 16 of 20 cases (80%) of B-CLLs studied by Mariano et al.32 Thus, it appears that mechanisms exist for achieving high levels of Bcl-2 protein accumulation in B-CLL cells that are independent of bel-2 gene rearrangements. Furthermore, based on our preliminary analysis of the in vitro survival of some representative cases of B-CLL, it appears that the high levels of Bcl-2 protein found in many CLLs are potentially of functional significance inasmuch as cells derived from a case of CLL with very low levels of Bcl-2 protein died with accelerated kinetics and experienced the DNA degradation, cell shrinkage, and morphologic changes typical of apoptosis more quickly than CLL cells that contained relatively high levels of Bcl-2 protein.

Precisely what these mechanisms are that lead to high levels of Bcl-2 protein remains to be determined, but it is of interest that DNA hypomethylation was observed in all 20 cases of B-CLL in a relatively selected region of the bel-2 gene corresponding to a 2.4-kb MspI restriction fragment that encompassed portions of the first major exon of this proto-oncogene. In most settings, hypomethylation is associated with upregulation of gene expression, or at least with the potential for induction of gene expression.53 In this regard, our analysis of t(14;18)-containing cell lines indicates that hypomethylation in the 5’-end of the bel-2 gene can be associated with transcriptional activation of this proto-oncogene. In all 6 of 6 t(14;18)-containing cell lines analyzed, for example, approximately 50% of the DNA corresponding to this 2.4-kb MspI fragment was cleavable by Hpa II based on scanning densitometry analysis of Southern blot autoradiograms (Fig 2 and data not shown). This result argues that one of the two copies of the bel-2 gene was hypomethylated in these lymphoma cell lines. Although not directly shown, presumably the hypomethylated copy represents the rearranged allele of bel-2 and not the germline form of the gene, because previous studies have shown that the unarranged bel-2 allele is transcriptionally silent in lymphoma cells that contain translocations involving bel-2.53,54 In contrast to t(14;18)-containing lymphoma cell lines, both copies of the bel-2 gene appeared to be hypomethylated in all 20 of the cases of B-CLL examined here. These data thus imply that the mechanisms accounting for the high levels of bel-2 gene expression in most cases of B-CLL are different from those involved when translocations affect one copy of this gene in lymphomas, and suggest that both bel-2 alleles may have the potential to become transcriptionally active in B-CLL cells. When taken together with the Southern blot data showing a lack of detectable bel-2 gene rearrangements, the DNA methylation results described here suggest that trans-regulatory mechanisms probably account for the high levels of bel-2 expression that occur in most cases of B-CLL, as opposed to the cis-regulatory defect embodied in the t(14;18) translocations of follicular lymphomas.

Although it is difficult to argue that DNA methylation directly controls the expression of bel-2 or other genes, all but 1 of the cases of B-CLL examined here (case no. 3) contained Bcl-2 protein at levels that were higher than in normal PBLs. Thus, in 19 of 20 cases, hypomethylation of the 5’-end of the bel-2 gene was associated with expression of this proto-oncogene. However, even if DNA methylation does directly contribute to the regulation of bel-2 expression in B-CLL, clearly other mechanisms must also be involved, given that the relative levels of Bcl-2 protein present in 19 cases of CLL that expressed bel-2 varied from 1.7 to 25 times the levels of Bcl-2 protein in normal PBLs.

Although the bel-2 gene is expressed in the majority of cases of B-CLL, at issue is whether this reflects an abnormality in the regulation of this proto-oncogene versus a reflection of a process that is normal for a particular stage of B-cell differentiation or for a subset of B cells. In a previous report in which normal CD5+ B cells were isolated from cord blood, bel-2 mRNA was found to be present at insufficient levels for detection by Northern blotting,53 suggesting that normal CD5+ cells do not express bel-2. In this case, the bel-2 expression seen in B-CLL could be regarded as pathologic. However, because CD5+ cord blood B cells exhibit an activated phenotype, the absence of bel-2 expression could also be a manifestation of other differences between the normal and neoplastic B cells. Alternatively, others have likened CD5+ B-CLL cells to the long-lived memory B cells that are located in the mantle zone of lymphoid follicles.51,55 Immunohistochemical studies of normal mantle zone B cells have shown intense immunoreactivity with anti–Bcl-2 antibodies, with the levels of immunostaining appearing subjectively at least to be equivalent to those found in t(14;18)-containing follicular lymphomas.56,57 Thus, the high levels of Bcl-2 protein that we detected in B-CLL cells may merely be a reflection of B cells that are committed to the memory pathway.

Although B-CLL cells are typically CD5+, we found no clear association of this leukocyte differentiation antigen with high levels of Bcl-2 protein, consistent with reports by others.31,32 The presence of PCNA protein also was not correlated either positively or negatively with Bcl-2 protein levels in our group of B-CLL samples. Previously, Schena et al.31 showed that stimulation of proliferation of certain B-CLL cell lines leads to downregulation of bel-2 gene expression. However, whereas most proliferating cells (as defined by expression of Ki67) lost immunodetectable Bcl-2 protein, some Ki67 cells with plasmacytoid features continued to express bel-2.51 When taken together with these data de-
rived from B-CLL cell lines, therefore, our data argue that bcl-2 expression is not necessarily inversely correlated with cellular proliferation in B-CLL.

The finding of high levels of Bcl-2 protein in the majority of B-CLL specimens has potentially important implications for better understanding the biology and clinical manifestations of this lymphoproliferative disorder. Similar to the cellular proliferation in B-CLL.

translocations involving bcl-2, B-CLL is characterized by a highly variable clinical course, with many patients enjoying normal age-adjusted survivals and others succumbing to their disease within 1 year of diagnosis. Although new therapies hold some promise of improved clinical outcomes, treatment in both diseases has traditionally been only palliative in nature, with death rates of 5% to 10% per year occurring essentially regardless of whether combination chemotherapy or single drug regimens are used. In both B-CLL and low-grade follicular lymphoma, the proportion of cells that are in cell cycle at any one time typically represents only a small percentage of the total, perhaps accounting in part for the current incurability of these B-cell malignancies. That bcl-2 is expressed at high levels in the majority of patients with low-grade non-Hodgkin B-cell lymphomas and B-CLL, however, suggests a direct connection between this proto-oncogene and the similar clinical behaviors of these disorders. Unlike other oncogenes described to date, the bcl-2 gene is unique in that the principal mechanism by which it contributes to neoplastic cell expansion is by prolonging cell survival rather than by accelerating the rate of cell proliferation. Molecular analysis of clinical materials as well as studies in transgenic mice that contain bcl-2/Ig fusion genes indicate that this survival advantage conferred by bcl-2 allows cells to accumulate due to a lack of normal turnover, and can set the stage for the acquisition of additional genetic lesions that can act in concert with bcl-2 and thus lead to more aggressive disease. Moreover, because overproduction of the Bcl-2 protein can also markedly increase the relative resistance of cells to killing by γ-irradiation and both cell-cycle-dependent and -independent chemotherapeutic drugs, the high levels of bcl-2 expression commonly found in B-CLL and low-grade follicular NHL cells may account in part for the difficulty in eradicating malignant cells from patients affected by these lymphoproliferative disorders.

REFERENCES

9. Tsujimoto Y, Louie E, Bashir MM, Croce CM: The reciprocal partners of both the t(14;18) and the t(11;14) translocations involved in B-cell neoplasms are rearranged by the same mechanism. Oncogene 2:347, 1988
14. Miyashita T, Reed JC: BCL-2 gene transfer increases relative resistance of S49.1 and WEHI17.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. Cancer Res 52:5407, 1992
23. Tsujimoto Y, Croce CM: Analysis of the structure, tran-
scripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 83:5214, 1986
42. Gauwerky CE, Hoxie J, Nowell PC, Croce CM: Pre-B-cell leukemia with a t(8;14) translocation is preceded by follicular lymphoma. Oncogene 2:431, 1988
bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia

M Hanada, D Delia, A Aiello, E Stadtmauer and JC Reed