Fatal Acute Lymphoblastic Leukemia in Mice Transgenic for B Cell-Restricted bcl-xL and c-myc

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Expression of the c-myc gene is frequently dysregulated in malignant tumors and translocations of c-myc into the Ig H chain locus are associated with Burkitt’s-type lymphoma. There is indirect evidence that bcl-x, an anti-apoptotic member of the bcl-2 gene family, may also contribute to a variety of B lymphoid tumors. In this study, we show that mice transgenic for both B cell-restricted c-myc and bcl-xL developed aggressive, acute leukemias expressing early B lineage and stem cell surface markers. Of interest, the tumor cells proliferated and differentiated down the B cell developmental pathway following in vitro treatment with IL-7. Analysis of sorted leukemic cells from spleen indicated constitutive expression of sterile μ and κ transcripts in combination with evidence for D-JH DNA rearrangements. Several B cell-specific genes were either not expressed or were expressed at low levels in primary tumor cells and were induced following culture with IL-7. IL-7 also increased V-Jc and V-DJH rearrangements. These data demonstrate oncogenic synergy between c-myc and bcl-xL in a new mouse model for acute lymphoblastic leukemia. Tumors in these animals target an early stage in B cell development characterized by the expression of both B lineage and stem cell genes. The Journal of Immunology, 2004, 172: 6684–6691.

In 1981, Hayward et al. (1) showed that avian leukemia virus-associated lymphomas were caused by retroviral insertion into the 5′ regulatory region of the c-myc gene. Shortly thereafter, Dalla-Favera et al. (2) demonstrated that c-myc was involved in the chromosomal (t8;14) translocations found in nearly all human sporadic and endemic Burkitt-type lymphomas (2), while Shen-Ong et al. (3) reported the involvement of myc translocations in mouse plasmacytomas (3). Since that time, there has been significant interest in understanding the mechanism by which Myc leads to cellular transformation (4).

Myc is a transcription factor of the B-zip helix-loop-helix family (5). Myc binds DNA E box elements containing the core sequence CAC(G/A)TG as an obligate heterodimer with Max, or other factors, and can function as either a transcriptional activator or repressor. The myc gene itself is transcriptionally up-regulated during normal proliferation and repressed in cells undergoing differentiation (6). Target genes for myc include ornithine decarboxylase (7) and the cell cycle phosphatase Cdc25A (8), either of which when overexpressed in cell culture at least partially mimic the transforming activity of Myc. Dysregulated expression of Myc causes cell cycle progression and proliferation, and, in addition, also induces apoptotic cell death (9). It has been proposed that these two functions of Myc, proliferation and apoptosis, are inherently linked processes (10). Dysregulated Myc expression along with blockade of Myc-induced death signaling, for instance by coexpression of the antiapoptotic gene bcl-2 (11, 12), is sufficient to induce malignant transformation of cells in a variety of systems.

Mice transgenic (Tg) for Myc within the B lineage (Eμ-mycc) have provided a useful model system to investigate the role of this oncogene in lymphomagenesis. Animals carrying the Eμ-mycc transgene harbor a large population of rapidly proliferating, but nontransformed, polyclonal pre-B and surface IgM+ B cells for the first several months of life (13, 14). This is followed by the subsequent development of highly malignant clonal B cell lymphomas that are invariably lethal, with mice dying between 4 and 7 mo of age. The clonal nature of the fatal lymphomas in these mice in combination with the variable timing of tumor onset is consistent with spontaneous secondary genetic mutations, and such mutations have been documented in ras, ARF, and p53 (15, 16). This model has also been used to show synergy between bcl-2 and c-myc in malignant transformation of B lineage cells (17, 18). Mice Tg for both a B cell-restricted bcl-2 transgene along with the Eμ-mycc transgene developed highly malignant lymphomas in the first few weeks of life. These tumors expressed markers of early hemopoietic cells and could be serially transplanted in recipient mice (17). Cell lines derived from these tumors were able to differentiate down either the B lymphoid or macrophage developmental pathways following appropriate stimulation with cytokines and/or LPS (18).

Bcl-x is a member of the bcl-2 family of death regulatory genes (19), and bcl-xL, the long isoform of the gene, has potent death inhibitory activity. Bcl-xL, like bcl-2, is thought to inhibit cell death by protecting the mitochondrial membrane from release of cytochrome c following various apoptotic stimuli (20). Bcl-xL prolongs the life span of transfected cells and renders such cells resistant to death following incubation with many inducers of apoptosis in vitro (19, 21). B and T cells from lymphocyte-targeted

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Received for publication May 7, 2003. Accepted for publication March 12, 2004.

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1 This work was supported by medical student fellowship awards from the National Arthritis Foundation and the Minnesota Medical Foundation (to P.J.S.) and National Institutes of Health Grant AR43805 and a Leukemia and Lymphoma Society award (to T.W.B.).

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3 Abbreviations used in this paper: Tg, transgenic; ALL, acute lymphoblastic leukemia; Rag, recombination-activating gene; SFF, specific pathogen free; PAS, periodic acid-Schiff, MN, Minnesota.
bcl-x<sub>L</sub> Tg mice also show resistance to cell death in vivo (22–24). For instance, Tg overexpression of bcl-x<sub>L</sub> in B cells leads to the accumulation of large numbers of pro-B cells with failed V(D)J rearrangements (22) and allows self-reactive B cells to survive in the presence of membrane-bound self-Ag (25).

Bcl-x<sub>L</sub> is overexpressed in several solid tumors (26–28) as well as hematopoietic tumors including acute myelogenous leukemia (29), Hodgkin’s lymphoma (30), non-Hodgkin’s lymphoma (31), HIV-associated lymphoma (32), and myeloma (33). Recently, translocations and rearrangements of bcl-x have been demonstrated in murine B and T cell tumor lines (34).

In the studies described here, we bred Eμ-myc Tg mice with animals overexpressing B cell-restricted Bcl-x<sub>L</sub>. Bcl-x<sub>L</sub>/mmyc double Tg animals developed highly malignant and fatal leukemia, with the transformed cells representing a very early stage in mouse B cell development. These mice provide a new model for the study of acute lymphocytic leukemia (ALL), and demonstrate oncogenic synergy between bcl-x<sub>L</sub> and c-myc.

Materials and Methods

Mice

Heterozygote bcl-x<sub>L</sub> B cell Tg mice (22) and Eμ-c-myc Tg mice (13), both fully backcrossed onto the C57BL/6 genetic background, were bred in the specific pathogen-free (SPF) animal facility at the University of Minnesota (Minneapolis, MN). Genotyping for the bcl-x transgene was performed as described previously (22). The c-myc transgene was detected by PCR using the forward primer 5'-GCAGAAGTCTGCAATTC-3' and the reverse primer 5'-GCGCGTATTAGCTAGCCGGTT-3'. Double Tg mice were sacrificed when they developed signs and symptoms of advanced malignancy (ruffled fur, growth arrest, tachypnea, hunched posture) and whenever possible were used immediately for experiments. All animals were housed in a SPF facility at the University of Minnesota, and all experiments were approved by the University of Minnesota Animal Care Committee.

Cell preparation

Bone marrow (BM), spleen, and blood were obtained as described elsewhere (25, 35). Briefly, single-cell suspensions of BM (from both femurs and tibias) were prepared by flushing the marrow cavities with wash buffer (1× RPMI 1640 with glutamate, 2.5% FCS). Spleen preps were obtained by gentle dissociation of whole spleens followed by filtering through a cell strainer. Peripheral blood was obtained by venous puncture or at autopsy, and RBC were depleted by ACK lysis.

Staining, flow cytometry, and cell sorting

The following directly conjugated mAbs were used for cell staining: B220-Cy, CD43-PE, Sca-1-FTTC, CD44-Cy, Gr-1-FTTC, Thy 1.2-PE, CD19-PE, IgM-PE, IgD-FTTC, CD34-FTTC, Mac-1-FTTC, CD34-PE (all Abs purchased from BD PharMingen, San Diego, CA). Standard flow cytometric analysis was conducted on a FACScalibur machine (BD Biosciences, Mountain View, CA) at the University of Minnesota Cancer Center, and data were analyzed using CellQuest (BD Biosciences) and FlowJo (Tree-star, Ashland, OR) software. For sterile sorting, splenic cells were stained with anti-B220, anti-Sca-1, and anti-CD43 mAbs, and samples were held at 4°C during the sterile sort. Reanalysis of sorted fractions showed purities in excess of 98%.

Serial transplantation

Splenics (10<sup>6</sup> in 100 μl of PBS) from a double Tg mouse (CD45.2 B6 allototype) were injected into the peritoneal cavity of CD45.1-congenic B6 recipient mice. Approximately 4–6 wk later the recipient animals showed signs of malignancy and were sacrificed for the next round of transplantation. The allototype difference allowed unequivocal identification of the transplanted tumor cells and was used for sorting of transplanted leukemic cells.

Cell culture

Splenics (≈90% leukemic) from c-myc/bcl-x<sub>L</sub> double Tg mice were cultured in 50% RPMI 1640/50% EHAA medium containing 10% heat-inactivated FCS (HyClone, Logan, UT), 50 μM 2-ME, 2 mM L-glutamine, and antibiotics at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells were cultured at an initial concentration of 0.5 × 10<sup>6</sup> cells/ml with 50 U/ml IL-7 (R&D Systems, Minneapolis, MN). Cultured cells were harvested between 12 and 18 days for flow cytometric analysis and preparation of mRNA and genomic DNA.

RT-PCR and PCR assays

All semiquantitative RT-PCR were validated and performed as previously described (22, 25, 36). Poly(A)<sup>+</sup> mRNA was extracted from 3 to 5 × 10<sup>6</sup> fresh sorted cells and from 5 × 10<sup>5</sup> IL-7 cultured cells using a MicroFast Track kit (Invitrogen, San Diego, CA). mRNA was stored as an ethanol precipitate at −80°C until ready for use. Two hundred nanograms of each cDNA sample was used for each semiquantitative PCR using gene-specific primer pairs (β-actin combination-activating gene (Rag) 1, Rag2, TdT, A5, VpreB (37); CD19 (38); sterile μ, sterile κ (39); B29, BSAP, Id, and E47 (40)). PCR was performed in 20-μl volumes containing 1 μl of cDNA, 1× PCR buffer, 2.5 mM MgCl<sub>2</sub>, 200 mM dNTPs, 100 ng of each sense and antisense primer, and 1 U of AmpliTaq Gold (PerkinElmer, Wellesley, MA). After an initial 10-min incubation at 95°C, the PCR was conducted as follows: denaturation at 95°C for 30 s, annealing at 60°C for 30 s (for the first five cycles) and 55°C (for subsequent cycles), and extension at 72°C for 45 s. Cycles (ranging from 22 to 30) were optimized so that amplification was in the linear range. The annealing temp was 64°C for the CD19 amplification and 60°C for sterile μ and sterile κ. Aliquots of the PCR samples were then electrophoresed through 1% agarose gels and blotted onto nitrocellulose membranes. Following UV cross-linking, the blots were hybridized overnight with either a randomly primed radiolabeled cDNA probe at 42°C (for β-actin, Rag1, Rag2) or a [γ-<sup>32</sup>P]ATP end-labeled oligonucleotide internal probe at 56°C. Hybridization probes used were as follows: TdT, AGC TGC AGA ACA TAA CAA CCA CCT GCT; A5, AGG CTA GAA TGA TGA ACT GCC AAG GAG G; VpreB, AGC TCT GCC AAC CAT AAT TG; CD19, TCC GTG GGC ATC TTC GTG GTT ATT G; sterile μ, AGC ACC ATT TTC TTC ACC TGG AAC T; sterile κ, TCC AGT GAG CAG TTA AAT GGA G; B29, TAC CAG CAA TGA CAA CCA GGA GTG ACC TTC; BSAP, GTC GCC CCG AAA AGG ATA GTG GAA CTT G; Id, GGT ACT TCT GTC GCA GCA AAG C; and E47, ACT CTT TGA CAC TAG TCA TGG GCT G. Following hybridization, membranes were washed and autoradiography. Genomic DNA was isolated from sorted tumor cells and in vitro-cultured cells using a high salt precipitation protocol. One hundred fifty nanograms of DNA (from ~40,000 cells) was used for each PCR. The DH-J2H, V-DJH2, V-Jk1, and control cκ assays were performed as previously described (22, 25, 36).

Results

Bcl-x<sub>L</sub> and c-myc synergize in leukemogenesis

Bcl-x<sub>L</sub> B cell Tg mice (22) have been observed up to 2 years of age and do not exhibit an accelerated incidence of lymphoid malignancies compared with non-Tg littermates. Thus, similar to bcl-2 B cell transgenics (41), the frequency of lymphomas in mice Tg for bcl-x alone appears to be low. To test the hypothesis that bcl-x<sub>L</sub> could synergize with c-myc to induce malignant transformation of B lineage cells, we initiated a series of breedings between bcl-x<sub>L</sub> and Eμ-myc Tg mice. All mice were on the C57BL/6 genetic background and were housed in SPF conditions.

Fig. 1 shows curves for the times to death or terminal disease for the non-Tg control littermates (B6), Bcl-x<sub>L</sub> single Tg (Bcl-x<sub>L</sub>), c-myc single Tg (Myc), and bcl-x<sub>L</sub>/c-myc (Bcl-x<sub>L</sub>/Myc) double Tg animals. There were no deaths of either B6 or bcl-x<sub>L</sub> Tg animals during the observation period. The median survival for c-myc Tg animals was 20 wk, with animals first developing a polyclonal expansion of slgM<sup>+</sup> pre-B cells and then succumbing to a clonal pre-B or mature B cell lymphoma, as previously described (13, 14). Strikingly, mice Tg both bcl-x<sub>L</sub> and c-myc had a significantly shortened median survival of only 6 wk compared with c-myc mice, with all of the double Tg animals dying before 10 wk of age. The double Tg animals could usually be identified by the second or third week of life due to their small size, a hunched posture, and ruffled fur.

Examination of blood, BM, and spleens from 4- to 6-wk-old bcl-x<sub>L</sub>/c-myc double Tg animals demonstrated the presence of an aggressive leukemia, with circulating leukemic cells in blood and
extensive leukemic infiltration of lymphoid organs (BM, spleen, lymph nodes). As shown in Fig. 2, the blood of bcl-xL/c-myc double Tg mice contained high numbers of lymphoid blasts (Fig. 2, A and B), many of which exhibited large cytoplasmic vacuoles (Fig. 2B). Of interest, similar vacuoles are often observed clinically in the leukemic cells of patients with Burkitt’s lymphoma (42), although classically Burkitt’s cells contain multiple small vacuoles. Staining of the leukemic cells from bcl-xL/c-myc mice with periodic acid-Schiff (PAS) revealed a “block-like” pattern of cytoplasmic staining in nearly all tumor cells (Fig. 2C, small arrows), a finding characteristic of human ALL (43).

The double Tg spleens were markedly enlarged (cf Fig. 2, D and G), and the normal architecture of the spleen was severely disrupted with poorly defined follicles and extensive infiltration of the red pulp with tumor cells (Fig. 2, D–F). There was also evidence in most mice for extramedullary hemopoiesis in the double Tg spleens characterized by the presence of megakaryocyte progenitors and myeloid blasts. Leukemic infiltrates were present in most internal organs examined (e.g., thymus, lung, liver, and kidney; data not shown), generally most prominent in perivascular regions. Visible tumor nodules were often observed on the surface of the liver. Morphologically, the leukemic cells were large cells containing prominent nuclei with a loose chromatin configuration (Fig. 2B). Only a thin margin of cytoplasm could be visualized in the tumor cells by light microscopy. These morphologic features are typical of human ALL (44).

Leukemic Bcl-xL/myc tumor cells are large and cycling
Flow cytometric examination of BM cells showed that the B220+ cells of both myc and bcl-xL/myc Tg animals were larger, as measured...
by forward light scatter, than those found in control or bcl-xL Tg animals (Fig. 3). The median size of B220<sup>+</sup> cells in bcl-xL/c-Myc Tg mice was slightly greater than myc-alone Tg cells. Examination of cell cycle status of BM B220<sup>+</sup> cells by propidium iodide staining showed that although ~10% of control B220<sup>+</sup> cells were in S or the G<sub>2</sub>-M phases of the cell cycle, 20% of bcl-xL Tg cells were in cycle, and 35 and 31% of myc and bcl-xL/myc double Tg cells, respectively, were cycling (Fig. 3). These results indicate that the double Tg tumor cells are rapidly cycling in vivo at rates similar to c-myc-alone Tg B cells.

**Cell surface phenotype of leukemic cells**

We next used flow cytometry to determine the cell surface phenotype of the leukemic cells in bcl-xL/myc double Tg animals. A representative experiment (of >20 performed) is shown in Fig. 4. The upper panel (Fig. 4) shows that the myc/bcl-x animals had a paucity of B220<sup>+</sup>IgM<sup>+</sup> B cells in BM, spleen, or blood, and instead exhibited a dominant population of IgM<sup>+</sup> cells. Furthermore, most of the leukemic cells expressed B220 and high levels of the stem cell marker Sca-1, whereas Sca-1<sup>+</sup> cells were very rare in any of the other mice.

Additional flow cytometric analysis revealed that the leukemic cells also expressed the B cell markers CD43 and AA4.1, the T cell markers CD4 and Thy1.2, and the myeloid marker Gr-1 (Fig. 5 and data not shown). The tumor cells did not express significant levels of CD19, IgM, IgD, CD3, Mac-1, CD34, c-kirR, or heat-stable Ag (Fig. 5 and data not shown). The tumors from >20 bcl-xL/c-myc mice examined exhibited a virtually identical cell surface phenotype. Together, these data indicate that within the first few weeks of life bcl-xL/c-myc double Tg mice develop a highly malignant and fatal leukemia, with the tumor cells expressing surface markers characteristic of several hemopoietic lineages.

**Serial transplantation of leukemic cells**

A series of experiments were next performed to determine whether the leukemic cells could be serially transplanted in syngeneic animals. The recipient mice used were congenic for the allotype marker CD45.1, so that the tumor cells (derived from CD45.2 allotype C57BL/6 mice) could be readily distinguished from host cells. Following i.p. injection of recipient mice with 10<sup>5</sup> or 10<sup>6</sup> cells.
spleenocytes, tumor cells were found to be easily transplantable. Recipients generally became ill with widespread leukemia of donor origin (CD45.2) within 4–7 wk, and the tumors could be serially transplanted for at least 12 generations. The cell surface phenotype of transplanted tumor cells was essentially indistinguishable from the parental tumor, with the exception that the transferred cells showed a slight and reproducible down-regulation in the expression of Sca-1 (data not shown).

**Tumor cells are IL-7 responsive**

Because of the high level expression of B220 and CD43 on the leukemic cells, we were interested in determining the ability of these cells to grow and differentiate in response to the B cell cytokine IL-7, known to be essential for normal B cell development and in particular for the normal proliferation and differentiation of early pro-B cells (40). In the absence of cytokine, tumor cells remained viable for ~3–4 wk in primary in vitro culture and showed a low basal level of proliferation (Fig. 5A). After 5 days in culture with IL-7, the tumor cells proliferated rapidly (Fig. 5A) and maintained survival for up to 6 wk (data not shown). Interestingly, the cell surface phenotype of the cells changed following culture in IL-7. IL-7-treated cells remained B220bright, CD43bright, and IgM+; however, CD19 levels were up-regulated in the cultured cells and the cells lost expression of Gr-1 and Sca-1 (Fig. 5B). This was consistent with differentiation down the B lineage pathway in response to IL-7.

**Gene expression and Ig rearrangements in IL-7-treated tumor cells**

To investigate gene expression in these tumors, purified populations of leukemic cells were sorted from the spleen cell suspensions of three double Tg mice, followed by mRNA and genomic DNA isolation. Sorting diminished the possibility of significant contamination of the cell preparations with nonleukemic B cells. Each of the three tumor populations was also cultured in vitro with IL-7, the tumor cells proliferated rapidly (Fig. 5A). After 5 days in culture with IL-7, the tumor cells proliferated rapidly (Fig. 5A) and maintained survival for up to 6 wk (data not shown). Interestingly, the cell surface phenotype of the cells changed following culture in IL-7. IL-7-treated cells remained B220bright, CD43bright, and IgM+; however, CD19 levels were up-regulated in the cultured cells and the cells lost expression of Gr-1 and Sca-1 (Fig. 5B). This was consistent with differentiation down the B lineage pathway in response to IL-7.

*FIGURE 6.* Induction of B cell-specific gene expression in tumor cells grown in IL-7. RT-PCR assays of gene expression in three fresh tumor samples (a, b, and c, lanes 3, 4, and 5, respectively) and in the same tumor samples after 12–16 days of culture with IL-7 (a', b', and c', lanes 6, 7, and 8, respectively). Lane 9 (d) shows quantitative RT-PCR analysis of a tumor that was serially transplanted through 10 recipient mice. The positive control (+, lane 2) for these experiments was normal BM, and the negative control (−, lane 1) was BM in which reverse transcriptase was eliminated from the reactions.

*FIGURE 7.* Induction of polyclonal Ig rearrangements in tumor cells cultured in IL-7. A, PCR assays to detect rearrangements at the H chain (D-JH and V-DJH) and L chain (V-Jkl) loci in three fresh tumor samples (a–c) and the same tumor samples after 12–16 days of culture with IL-7 (a', b', and c'). The last lane (d) shows RT-PCR analysis of a tumor that was serially transplanted through 10 recipient mice. The control PCR is a portion of the constant region in theκ locus (Cκ). Positive control (+, lane 2), Normal BM; negative control (−, lane 1), BM, no reverse transcriptase in the reactions. B, V-DJH2 joints were amplified from normal B6 spleen, fresh tumor cells, and IL-7-cultured tumor cells. Control in the upper panel is genomic DNA from mouse tail. The detected rearrangements in spleen shows a pattern of peaks separated by 3 bp, reflecting the selection for in-frame and productive V-DJH2 joints. In contrast, the pattern in the cultured tumor cells does not show the triplets, indicating a lack of selection for productive joints, as expected.

IL-7 for 12–16 days before harvesting for mRNA and genomic DNA isolation and flow cytometric analysis. Semiquantitative reverse transcription and genomic PCR were then performed on the fresh and cultured samples, as well as on serially transplanted tumor cells at passage 10 (sorted based on B220, CD45.2, and CD43 staining). Cycle numbers were adjusted so that amplifications were in the linear range (generally between 22 and 30 cycles), and blotted PCR products were hybridized with radiolabeled internal oligonucleotides (36). The results of these analyses are shown in Figs. 6 and 7.

Fresh tumor cells (Fig. 6, a–c, lanes 3–5, respectively) expressed low levels of Rag2 and Rag1, TdT, the surrogate L chains...
VpreB and A5, the B cell surface molecule CD19, and the B cell transcription factor BSAP. Each of these genes was induced following culture with IL-7 (Fig. 6, a’, b’, and c’, lanes 6–8, respectively). The transcription factor E47 and the Ig receptor-associated signaling molecule Igβ were expressed at equivalent levels in all samples. Sterile μ (μc) transcripts, which indicate transcriptional activity at the unrearranged Ig H chain loci, were observed in fresh tumor samples and were down-regulated following culture in IL-7. Sterile κ (κc) transcripts did not change. The RT-PCR results in the serially transplanted tumor sample (Fig. 6d, lane 9) generally mirrored the findings in the fresh tumor samples. Although low levels of CD19 mRNA were detected in fresh tumor, there were only background levels of surface CD19 staining by flow cytometry (see Fig. 5B).

The status of Ig H and L chain DNA rearrangements in the tumor cell populations was then examined by PCR of genomic DNA. DJ1, DJ2, and V-DJ DNA rearrangements were identified at significant levels in both fresh and cultured cells (Fig. 7A, upper panel), while V(D)J and V-Jκ1 rearrangements were detected at low levels in the fresh tumor cells and were induced in cultured cells. Despite the evidence for Ig rearrangements in both fresh and cultured tumor cells, we were not able to demonstrate surface or intracellular expression of IgM (Fig. 5B and data not shown). This may reflect either a very low frequency of productive rearrangements or it remains possible that other genes required for the assembly and cell surface expression of IgM may not be expressed normally in these tumor cells.

The finding of rearrangements at both the Jκ1 and Jκ2 loci in the DJκ1 and V-DJκ2 DNA rearrangements were identified at significant levels in both fresh and cultured cells (Fig. 7A, upper panel), while V(D)J and V-Jκ1 rearrangements were detected at low levels in the fresh tumor cells and were induced in cultured cells. Despite the evidence for Ig rearrangements in both fresh and cultured tumor cells, we were not able to demonstrate surface or intracellular expression of IgM (Fig. 5B and data not shown). This may reflect either a very low frequency of productive rearrangements or it remains possible that other genes required for the assembly and cell surface expression of IgM may not be expressed normally in these tumor cells.

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The finding of rearrangements at both the Jκ1 and Jκ2 loci in the DJκ1 and V-DJκ2 assays (Fig. 7A) suggested the possibility that the tumors in these animals were polyclonal. To address the issue of clonality more definitively, we amplified genomic DNA from sorted fresh and cultured tumor cells with V-DJκ2 primers and visualized the fluorescently labeled products on a denaturing sequencing gel. As shown in Fig. 7B, the V-DJκ2 joints of tumor cells cultured with IL-7 were of multiple lengths, indicating the polyclonal nature of the tumors in double Tg mice.

Discussion

The data presented demonstrate that bcl-xL can cooperate with c-myc in the malignant transformation of early B cell progenitors. Mice Tg for cell-restricted bcl-xL and c-myc transgenes developed highly malignant and fatal ALL. The median survival of double Tg animals was only 6 wk and no animals lived longer than 10 wk. Fresh tumor cells from these mice expressed the cell surface markers B220, CD19, and Sca-1 and were highly responsive to a single cytokine (IL-7) in the absence of stromal support.

It was of interest, then, that bcl-xL-SV40/myc double Tg mice developed fatal tumors that were essentially undistinguishable from those in bcl-xL-MN/myc mice (data not shown). The animals had a similar short life span, and fresh tumor cells showed an identical surface phenotype. Bcl-xL-SV40/myc tumors also proliferated and progressed down the B lineage in response to IL-7 similar to bcl-xL-MN/myc tumors (data not shown). Thus, the two bcl-xL transgenes were equally capable of synergizing with myc to induce transformation of an early B cell precursor cell, despite the fact that the bcl-xL-SV40 transgene is likely expressed at significantly lower levels in early B cell precursors than the bcl-xL-MN transgene.

One of the most striking features of the tumors that develop in bcl-xL/myc or bcl-2/myc animals is the very primitive cell type that is targeted for malignant transformation. It is possible that the normal counterpart of these tumor cells is particularly susceptible to transformation by the combination of myc with either bcl-xL or bcl-2. It is also possible that this early stage in B cell development is uniquely susceptible to the model because of the nature of the Tg expression constructs used to drive expression of the transgenes. Expression of both the bcl-xL and c-myc transgenes is controlled by the Ig HC intronic enhancer, and the cell type targeted in these tumors is very close to the stage of B cell development where the HC intronic enhancer is first expressed at high levels. These tumors express a number of cell surface markers and genes (e.g., B220, CD19, sterile μ, and κ transcripts, Rag1, Igβ) characteristic of the earliest stages of pro-B cell development. The tumors in addition expressed Thy1.2, Gr-1, and Sca-1, consistent with a more primitive progenitor phenotype. This tumor cell phenotype may represent a low abundance population present in normal BM or the cells may express their unique combination of markers due to the effects of constitutive myc and bcl-x expression.

The tumors that arise in bcl-xL/myc double Tg mice are similar in some ways to the tumors previously described in bcl-2/myc double Tg animals (17, 18). Fresh tumor cells from bcl-xL/myc and bcl-2/myc animals have a similar cell surface phenotype. Bcl-2/myc tumors were B220+, CD43−, Sca-1−, Gr-1−, Thy-1−, and CD44−, virtually the same suite of markers expressed on bcl-xL/myc cells. The time course of tumor progression in the two models was also similar, bcl-2/myc mice lived only ~6–8 wk.

The tumors that form in bcl-xL/myc and bcl-2/myc mice also appear to differ in several significant ways. Bcl-2/myc tumors were reported to be very difficult to culture in vitro, with cells dying after only 1 or 2 days of in vitro culture (18). Although cell lines were eventually established from bcl-2/myc animals, their establishment required incubation with multiple cytokines along with growth on a stromal layer. In contrast, bcl-xL/myc tumor cells have a relatively prolonged in vitro life span (days to weeks) and were highly responsive to a single cytokine (IL-7) in the absence of stromal support.

Several factors may contribute to the different phenotypes observed between the two models. This could reflect important functional differences between the bcl-xL and bcl-2 proteins during early B cell development, as suggested by the differing phenotypes of knockout animals lacking bcl-xL or bcl-2 (47, 48). Bcl-xL knockout mice have very impaired early development of B cells, whereas bcl-2 knockout mice show relatively normal initial development of B cells but then a dramatic loss of survival of naive splenic B cells. The knockout phenotypes are consistent with the idea that bcl-xL may have a more important role in controlling survival early in B cell development than bcl-2.

The differing phenotypes of bcl-xL/myc and bcl-2/myc tumor cells might also reflect differing availability of key regulatory
dimerization partners (such as bax and bad) in early B lymphoid progenitors and/or subtle differences in the stage of development in which the transgene is expressed due to the different Tg constructs used to drive expression. Finally, bcl-x<sub>i</sub>/myc tumor cells, but not bcl-2/myc tumors, may be producing an autocrine cytokine (e.g., IL-7) or have other changes in gene expression that support improved survival or IL-7 responsiveness. Further studies will be required to establish the precise nature of these differences.

The IL-7 responsiveness of the bcl-x<sub>i</sub>/myc tumor cells was impressive. IL-7 is known to be a critical and nonredundant growth and differentiation factor for mouse B lymphocytes, and mice with targeted deletions of the IL-7 gene or of the IL-7Rα exhibit arrested B cell development at an early B cell stage of development (49). These data provide a striking example of how a tumor can retain cytokine responsiveness, which may contribute to its in vivo malignant behavior.

Recent data indicate that bcl-x<sub>i</sub> expression is normally suppressed by c-myc in primary myeloid and pre-B cells (50). Bcl-2 and bcl-x<sub>i</sub> were also found to be markedly suppressed in the pre-cancerous cells of Eμ-myc Tg B cells. Of interest, more than one-half of the spontaneous lymphomas that arise in Eμ-myc Tg mice overexpress either bcl-2 and/or bcl-x<sub>i</sub> (50). The triggers for induction of bcl-2 or bcl-x<sub>i</sub> in this model system are not yet known. However, several well-described oncogenes (Pim-1, c-myb, BCR-ABL) are known to up-regulate bcl-2, and the NF-κB pathway, which is turned on in many tumors, is a strong stimulus for bcl-x<sub>i</sub> transcription (51). The ability of myc to drive cell proliferation and at the same time deliver a potent death signal may explain why atypical apoptotic genes like bcl-2 and bcl-x<sub>i</sub> can synergize so potently with myc in malignant transformation.

Acknowledgments
We thank Jerry Adams for originally providing the c-myc Tg mice.

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