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Genetics

Growth- and tumor-promoting effects of deregulated *BCL2* in human B-lymphoblastoid cells

(lymphoma/oncogene/retroviral vector)

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ABSTRACT **Human follicular B-cell lymphomas possess a t(14;18) that translocates a putative protooncogene, *BCL2*, into the immunoglobulin heavy chain locus. The normal *BCL2* gene is quiescent in resting B cells, expressed in proliferating, but down-regulated in differentiated B cells. Inappropriately high levels of *BCL2*–immunoglobulin chimeric RNA are present in t(14;18) lymphomas for their mature B-cell stage. We examined the biologic effects of *BCL2* deregulation in human B cells by introducing *BCL2* into human B-lymphoblastoid cell lines (LCLs) with retroviral gene transfer. Although deregulated *BCL2* expression as a single agent was not sufficient to confer tumorigenicity to LCLs, it consistently produced a 3- to 4-fold increment in LCL clonogenicity in soft agar. In addition, *BCL2* deregulation complements the transforming effects of the *MYC* oncogene in LCLs. *BCL2* augmented the clonogenicity of LCLs bearing exogenous *MYC* and increased the frequency and shortened the latency of tumor induction in immunodeficient mice. These results demonstrate a role for *BCL2* as a protooncogene that affects B-cell growth and enhances B-cell neoplasia.**

The first interchromosomal translocation to be extensively characterized juxtaposed a known cellular oncogene *MYC* with the immunoglobulin locus in both Burkitt lymphomas and mouse plasmacytomas (1, 2). Activated *MYC* genes proved capable of inducing B-cell tumors in transgenic mice (3–5) or of transforming cultured human B-cell lines. A second generation of chromosomal translocations found in lymphoid neoplasms introduce putative protooncogenes into either the immunoglobulin or T-cell receptor loci. The best characterized of these is the human t(14;18) (q32;q21) found in ≥85% of follicular small cleaved B-cell lymphomas and in ≈20% of diffuse lymphomas (6, 7). No equivalent translocations have yet been noted in murine tumors. The t(14;18) appears to occur early in pre-B-cell development placing the *BCL2* gene into the immunoglobulin heavy chain (*IGH*) gene locus (8, 9). This creates a *BCL2–IGH* fusion gene, although the *BCL2* protein encoding region is not interrupted (8–13). The translocated *BCL2* is deregulated resulting in inappropriately high levels of BLC2–IGH chimeric mRNA in these mature B-cell tumors (13, 14).

The elevation of BCL2–IGH RNA and the lack of extensive deletion of chromosome 18 material argued that activation of *BCL2* is the predominant result of the t(14;18). However, neither the normal function nor the biologic effects of *BCL2* deregulation is precisely known. Since the t(14;18) is found almost exclusively in B-cell tumors, we chose a human mature B-cell system to assess the effects of deregulated *BCL2*. We used a retroviral vector to introduce *BCL2* into Epstein–Barr virus (EBV)-immortalized lymphoblastoid cell lines (LCLs). These cells represent a convenient transformation system to study the effects of oncogenes in human B cells as shown for *MYC* (3) and *RAS* (15, 16). Moreover, *BCL2* could be assessed within LCLs already expressing deregulated *MYC* to examine oncogene complementation and thus extend to human B cells the reported effects of *BCL2* upon cells from Eμ-*myc* transgenic mice (17). The data indicate a consistent effect of *BCL2* activation upon human B-cell growth and support its role in neoplasia.

METHODS

Cell Lines. The ecotropic (ψ2) and amphotropic (PA317) retrovirus packaging lines (18, 19) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) calf serum or 10% (vol/vol) fetal calf serum (FCS), respectively. LCLs, CB33, UH3, and CPH350.1, were derived from EBV-infected normal human peripheral blood or umbilical cord mononuclear cells (3). CB33SVmyc2,3 represents a LCL transfected with the human MYC expression plasmid (pHEBoSVmyc2,3) followed by hygromycin selection; it expresses exogenous MYC transcripts (3). All LCLs were maintained in Iscove's modified Dulbecco's medium (IMDM) containing 10% FCS.

N2-H–BCL2 Retroviral Vector Construction. The plasmid pN2 was provided by E. Gilboa (20). A 0.4-kilobase (kb) *Hin*dIII–*Kpn* I fragment containing the simian virus 40 (SV40) early promoter/enhancer region was inserted into the plasmid Bluescript (Stratagene). The resulting plasmid was cut with *Eco*RI to accommodate the 1.9-kb *Eco*RI human BCL2 cDNA fragment 58 (13). A 2.4-kb *Kpn* I–*Not* I fragment containing the SV40–*BCL2* fusion gene was excised from the plasmid polylinker, ligated to *Xho* I linkers, and inserted in the correct orientation into the *Xho* I cloning site of pN2 (Fig. 1).

Recombinant Retrovirus Packaging and Infection. Both ecotropic and amphotropic retroviruses were produced. pN2 or pN2-H–BCL2 plasmid DNA (12 μg) was first introduced into the PA317 amphotropic packaging line by calcium phosphate precipitation, and 2 days later the culture supernatant was used to infect the ψ2 ecotropic packaging line in the presence of Polybrene (5 μg/ml) (Sigma), as described by Miller *et al.* (21). The amphotropic retroviruses used here were produced by infecting 4×10^6 PA317 cells with 5.0 ml of high-titer supernatant from ψ2 cloned lines bearing N2 or N2-H–BCL2 in the presence of Polybrene. Forty-eight hours later G418 was added and cells were cultured in G418 for 12 days for selection. PA317 cell clones producing high-titer N2-H–BCL2 or N2 ($>5 \times 10^5$ colony-forming units/ml) were

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Abbreviations: LCL, lymphoblastoid cell line; FCS, fetal calf serum; EBV, Epstein–Barr virus; SV40, simian virus 40.

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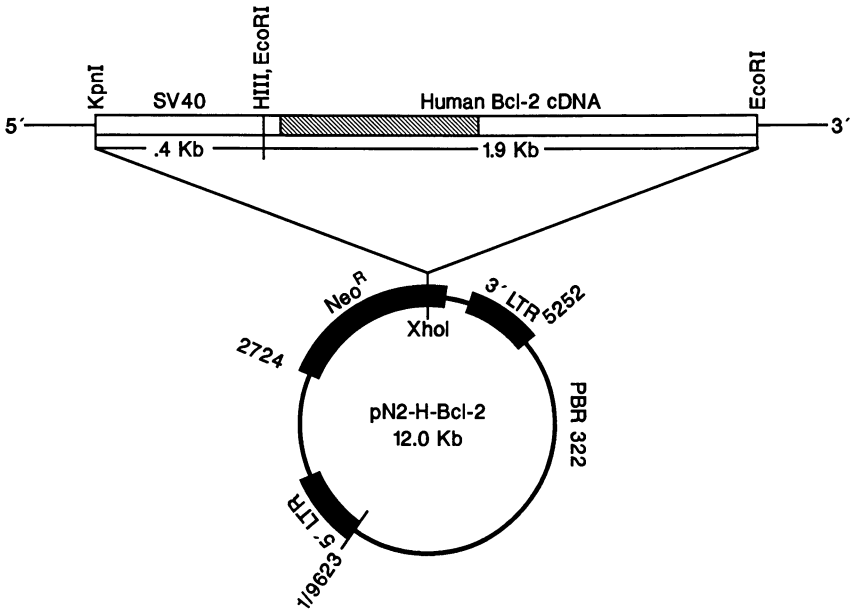

FIG. 1. Retroviral expression vector for human *BCL2* (protein encoding region cross-hatched) was created by insertion into the *Xho* I site of the defective retroviral vector pN2. A 1.9-kb *Eco*RI fragment of human BCL2 cDNA (fragment 58) from SU-DHL-6 was ligated at a *Hin*dIII polylinker site to the 0.4-kb *Kpn* I–*Hin*dIII SV40 early promoter/enhancer region. *Xho* I linkers were added and the correct orientation of pN2-H–BCL2 was selected. LTR, long terminal repeat; Neo^R, neomycin resistance.

identified by titering on NIH 3T3 cells. Bulk lines of retrovirally infected LCLs were produced by incubating 5×10^6 cells with virus supernatant in the presence of Polybrene (10 μg/ml) for 3 hr, followed by G418 selection (0.6 mg/ml). Southern blot analysis of DNA from retrovirus-infected cells showed integrated proviruses with intact *BCL2* and neomycin-resistance genes.

Northern Blot and RNase Protection Analysis. Total RNA was extracted by the guanidine thiocyanate method. For Northern blot analysis, RNA samples were electrophoresed in 0.9% agarose/2.2 M formaldehyde gels and then transferred to nitrocellulose filters. Hybridization and washes were performed as described (3). For RNase protection analysis, a uniformly labeled antisense RNA probe was prepared from a SV40–*BCL2* DNA template cloned into Bluescript using T7 RNA polymerase and [^{32}P]CTP, as described by Melton *et al.* (22). Labeled transcripts were hybridized to 20 μg of total RNA for 12 hr at 65°C and then digested with RNase A (40 μg/ml) and RNase T1 (2 μg/ml). Protected fragments were size-separated on denaturing 6% polyacrylamide gels and autoradiographed.

Clonogenicity in Agar. A low-sensitivity agar cloning assay was performed in triplicate by embedding 10^3–10^4 LCL cells in 1 ml of IMDM containing 20% FCS and 0.3% soft agar (Difco) in 30-mm plates. Colonies were scored at day 10. We also developed a high-sensitivity agar cloning assay. In this assay, 10^5 early passage human foreskin fibroblasts were plated in 30-mm plates and overlaid with 0.5% agar in IMDM plus 20% FCS. LCL cells (10^3–10^4 cells) were embedded in an overlayer of 0.3% agar in IMDM and 20% FCS. Colonies were scored at day 22. Both clonogenicity assays demonstrated a linear relationship between colony formation and the number of cells embedded in agar. Therefore, cloning efficiency was calculated using all data points.

Tumorigenicity in Nude Mice. Swiss female athymic nude mice (3–4 weeks old) were injected subcutaneously with cyclophosphamide [0.01 mg/g (body weight)]. Seventy-two hours later, mice were injected subcutaneously at two sites with 10^6, 3×10^6, or 5×10^6 washed viable cells. Animals were monitored for the appearance of tumors for at least 7 weeks. Tumors were considered present if at least 10 mm × 10 mm in size. Tumor diagnosis was confirmed by histological examination.

RESULTS

Retroviral Introduction of Deregulated *BCL2* Gene. EBV-immortalized LCLs (CPH305.1, UH3, and CB33) and LCLs possessing a MYC expression plasmid (CB33SVmyc2,3) were infected with N2 or N2-H–BCL2 high-titer virus supernatants and selected in G418. Northern blot analysis of all infected LCLs revealed the expression of retrovirally encoded neomycin-resistance and *BCL2* genes. Representative Northern blots of uninfected UH3 LCLs and UH3 cells infected by N2 (UH3.N2) demonstrate the low-level expression of the endogenous ≈6.5- and 4.0-kb BCL2 transcripts (Fig. 2*A*). N2-H–BCL2-infected cell lines demonstrate large amounts of exogenous 5.8- and 4.5-kb BCL2 transcripts as represented by UH3.BCL2 in Fig 2*A*. An RNase protection assay performed on N2-H–BCL2-infected LCL lines confirmed the presence of *BCL2* transcripts originating from both the SV40 initiation site and the 5′ long terminal repeat of the vector (Fig. 2*B*).

Expression of Deregulated *BCL2* Increases the Clonogenicity of LCLs. The growth effect of N2-H–BCL2 versus N2 viruses upon LCLs was assessed in a soft agar clonogenicity assay. A high-sensitivity agar cloning assay revealed that deregulated expression of *BCL2* alone was sufficient to increase the cloning efficiency of LCLs. In this system, 10^5 early passage human foreskin fibroblasts were used as a feeder layer. A representative assay for LCL CB33 possessing N2 versus N2-H–BCL2 is shown in Fig. 3. A 3- to 4-fold increment in colonies at day 22 was consistently seen in all LCLs carrying deregulated *BCL2* genes (Table 1). The inherent cloning efficiency of LCLs with either N2 or N2–BCL2 required a feeder layer for detection, as neither the low-sensitivity agar assay (Table 2) nor a limiting dilution liquid culture assay (data not shown) was sensitive enough to assess colony formation. No morphologic changes were noted in N2–BCL2-infected LCLs. The immunophenotypes of LCLs possessing N2-H–BCL2 versus N2 were also compared by flow cytometry (data not shown). No significant changes were

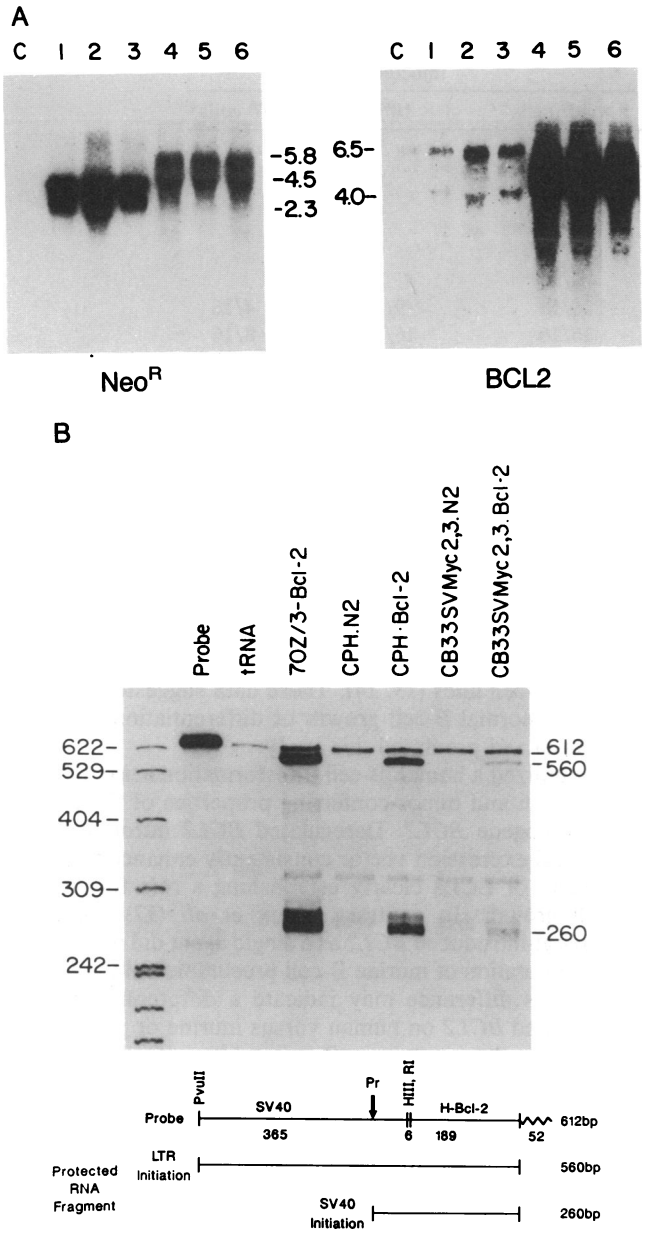

FIG. 2. (*A*) Expression of retrovirus-encoded genes in LCLs. RNAs from UH3 cells (lanes C), N2-infected UH3 cells (UH3.N2, lanes 1, 2, and 3), and N2-H–BCL2-infected UH3 cells (UH3.BCL2, lanes 4, 5, and 6) were hybridized with a neomycin-resistance gene probe (Neo^R) and a *BCL2* 1.5-kb *Hin*dIII–*Eco*RI exon III probe. Analysis of *BCL2* expression reveals that both the UH3 uninfected and the UH3.N2 cell lines express endogenous 6.5- and 4.0-kb transcripts, and the UH3.BCL2 cell lines express in addition large amounts of exogenous transcripts of 5.8 and 4.5 kb. (*B*) An RNase protection assay was performed on N2- and N2-H–BCL2-infected, G418-resistant LCLs, CPH350.1 and CB33SVmyc2,3. A murine 70z/3 line infected with N2-H–BCL2 and known to highly express N2-H–BCL2 served as a positive control. The full-length 612-base pair (bp) uniformly labeled RNA probe discriminates 560-bp transcripts originating from the long terminal repeat (LTR) from the 260-bp RNA initiated at the SV40 promoter. Sizes in bp are shown.

noted in B1 (CD20), B4 (CD19), plasma cell antigen 1 (PCA-1), B7 (CD21), M1 (CD11b), LeuM5 (CD11c), HC-2, LFA-1 (CD11a), T9 (Tfn-R), T10 (CD38), interleukin 2 receptor (CD25), BA-1 (CD24), BA-2 (CD9), common acute lymphoblastic leukemia antigen (CALLA or CD10), intercellular cell adhesion molecule (ICAM), β_2-microglobulin, HLA-DR, HLA-DQ, or HLA-DP (23, 24).

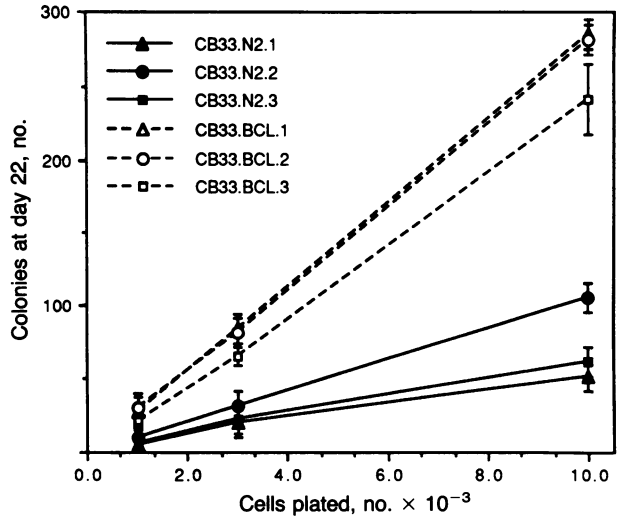

FIG. 3. Clonogenicity in agar of three CB33 lines containing either retrovirus-encoded N2- or N2-H–BCL2 genes (as indicated). CB33 cells were embedded in 0.3% agar at 10^3, 3×10^3, and 10^4 cells per dish on top of 10^5 early passage human foreskin fibroblasts in 0.5% agar. Data points represent the mean ± 1 SD of triplicate cultures.

Deregulated *BCL2* Complements *MYC* to Augment Clonogenicity in LCLs. A CB33 LCL (CB33SVmyc2,3) transfected with a MYC expression plasmid was infected with N2 and N2-H–BCL2. Since the inherent cloning efficiency of CB33SVmyc2,3 was >50% in the high-sensitivity agar cloning assay with a feeder layer (data not shown), effects of *BCL2* upon CB33SVmyc2,3 clonogenicity were assessed in the low-sensitivity agar assay. The average of the data performed in triplicate for four independently derived lines is shown in Table 2. The addition of a deregulated *BCL2* results in a modest but uniform 2-fold increment in clonogenicity in all the LCLs tested.

***BCL2* Enhances Tumorigenicity in Nude Mice.** Injection of nude mice with LCLs bearing a deregulated *BCL2* gene (5×10^6 cells per site) as a single agent resulted in no tumor formation (Table 2). Three lines, CPH350.1.BCL2, CB33.BCL2, and UH3.BCL2, all expressed exogenous *BCL2* and demonstrated increased agar clonogenicity but failed to establish tumors in immunodeficient mice.

We next examined whether deregulated *BCL2* would augment the tumorigenicity of LCL already transformed with a MYC expression vector. CB33SVmyc2,3 cells uniformly produce tumors in nude mice at 5×10^6 cells per site when

Table 1. Clonogenicity of LCLs infected with N2 and N2-H–BCL2

Cell line	Cloning efficiency, %
CB33.N2.1	0.48 ± 0.19
CB33.N2.2	0.89 ± 0.15
CB33.N2.3	0.60 ± 0.13
CB33.BCL.1	2.67 ± 0.46
CB33.BCL.2	2.99 ± 0.42
CB33.BCL.3	2.60 ± 0.51
UH3.N2.1	1.81 ± 0.50
UH3.N2.2	1.50 ± 0.29
UH3.N2.3	1.85 ± 0.19
UH3.BCL.1	4.39 ± 0.96
UH3.BCL.2	5.47 ± 1.15
UH3.BCL.3	4.16 ± 1.20

Each cell line is an independently derived LCL from a different infection. Cloning efficiency data represent the mean ± 1 SD of triplicate cultures using 10^3, 3×10^3, and 10^4 LCLs in the high-sensitivity agar cloning assay.

Table 2. Tumorigenicity in immunodeficient nude mice of different LCL populations

Cell line	Cloning efficiency, %	Number of tumors observed/number of sites injected		
		5×10^6 cells*	3×10^6 cells*	10^6 cells*
CPH350.1N2	0	0/6		
CPH350.1.BCL2	0	0/6		
CB33.N2	0	0/16		
CB33.BCL2	0	0/16		
UH3.N2	0	0/16		
UH3.BCL2	0	0/16		
CB33SVmyc2,3.N2	3.9 ± 1.2	16/16	9/16	4/16
CB33SVmyc2,3.BCL2	8.0 ± 1.2	16/16	16/16	8/16

Data from each cell line represent results from four independently derived LCLs except data for CPH350.1N2 and CPH350.1.BCL2, which are from a single cell line. Cloning efficiency data represent the mean ± 1 SD from triplicate cultures using 10^3, 2.5×10^3, 5×10^3, and 10^4 LCLs in the low-sensitivity agar cloning assay. Nude mice were monitored for at least 7 weeks.
*Number of cells injected.

compared to parent CB33 cells, which fail to do so (3). However, the addition of N2-H-BCL2 to such cells produced a consistent effect. A shortening in the latency period before tumor appearance was noted with CB33SVmyc2,3.BCL2 cells when compared to CB33SVmyc2,3.N2 (Fig. 4). Moreover, the expression of exogenous *BCL2* increased the incidence of tumors from 25% to 50% at 10^6 injected cells per site and from 56% to 100% at 3×10^6 cells per site (Fig. 4 and Table 2). These results indicate that although *BCL2* expression *per se* was not sufficient for tumorigenic conversion of LCLs, it enhanced tumor formation in EBV/*MYC*-transformed cells.

DISCUSSION

The molecular hallmark of follicular B-cell lymphoma is the t(14;18) that juxtaposes the *BCL2* gene with the immunoglobulin locus resulting in a deregulated *BCL2–IGH* fusion gene. The normal *BCL2* gene is highly expressed at the pre-B-cell stage of development, when this translocation appears to occur. Similar to the expression pattern of some oncogenes, *BCL2* proved to be quiescent in resting normal B cells and up-regulated with B-cell activation (14, 25). However, *BCL2* is down-regulated in differentiated B cells and mature-B-cell lines (13, 14). These data suggested a role for *BCL2* in normal B-cell growth or differentiation as well as neoplasia and prompted this study.

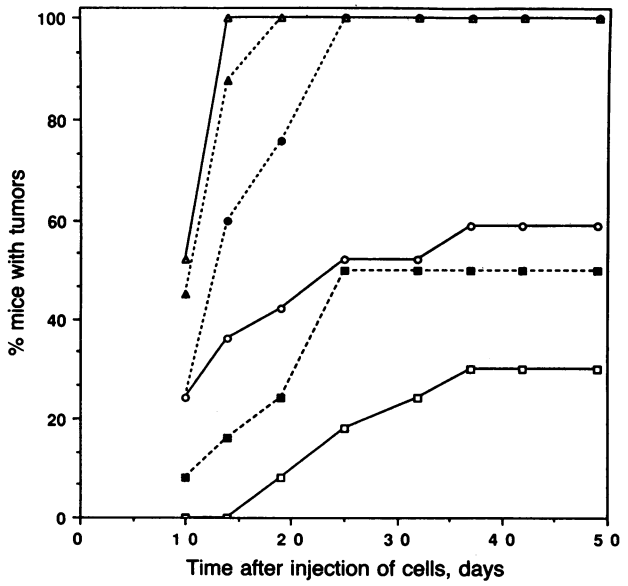

FIG. 4. Development of tumors in immunodeficient nude mice. Swiss female nude mice (3–4 weeks old) were injected with cyclophosphamide [0.01 mg/g (body weight)], and 72 hr later, mice were injected at two sites with the following cells. ▲, CB33SVmyc2,3.BCL2 at 5×10^6 cells per site; ●, CB33SVmyc2,3.BCL2 at 3×10^6 cells per site; ■, CB33SVmyc2,3.BCL2 at 10^6 cells per site; △, CB33SVmyc2,3.N2 at 5×10^6 cells per site; ○, CB33SVmyc2,3.N2 at 3×10^6 cells per site; □, CB33SVmyc2,3.N2 at 10^6 cells per site. Animals were monitored for appearance of tumors for at least 7 weeks. Tumors were scored positive if at least 10 mm × 10 mm in size.

We utilized a human B-cell transformation assay to assess the growth and tumor-conferring properties of the putative protooncogene *BCL2*. Deregulated *BCL2* introduced by a retroviral expression vector consistently enhanced the clonogenicity of LCLs clearly establishing a role for *BCL2* in B-cell growth. In contrast, Vaux *et al.* (17) noted that retroviral-introduced *BCL2* as a single agent did not promote the proliferation of murine B-cell precursors cultured in soft agar. This difference may indicate a differential effect of deregulated *BCL2* on human versus murine or perhaps mature rather than immature B cells. Alternatively, although *BCL2* will complement the effects of the EBV genome in LCLs, it may not be capable of inducing proliferation as a single agent. Consistent with a limited effect for *BCL2*, we observed no tumor formation in immunodeficient mice when *BCL2* alone was introduced into LCLs nor was the serum dependence of such lines altered. We obtained similar results with an additional series of pHEBo vectors placing *BCL2* under SV40 and cytomegalovirus promoters (data not shown).

The transfection of LCLs with pHEBoSVmyc2,3 followed by selection with hygromycin (3) allowed the secondary addition of retroviral *BCL2* and its selection with G418. *BCL2* proved capable of augmenting the effect of a deregulated *MYC* as assessed by an improvement in clonogenicity and an increased frequency and shortening of the latency of tumor induction in immunodeficient mice. Although *BCL2* will complement *MYC*, this interaction is not markedly synergistic, perhaps indicating that the *MYC* oncogene improves the threshold of tumorigenicity to enable the effects of *BCL2* deregulation to be quantitated. This interaction between *BCL2* and *MYC* oncogenes provides a model for a subset of human lymphomas that activate both genes. These B-lineage tumors possess two distinct translocations involving *MYC*, t(8;14) as well as *BCL2*, t(14;18) and display a much more aggressive clinical course in comparison to lymphomas with only the t(14;18) (26–28). Moreover, a composite lymphoma illustrated the progression of a t(14;18) tumor by the addition of a *MYC* rearrangement in the most abnormal regions of a lymph node (29).

Although the data presented here clearly establishes that *BCL2* can affect the growth of human B cells, the biochemical mechanism is unknown. *BCL2* has no close homology with other genes and encodes a 25-kDa protein that appears to be intracellular and membrane associated (30). *BCL2* had no consistent effect upon the differentiation stage of LCLs as assessed by cell surface antigens. Beyond the B-cell lineage, we assessed the effects of N2-H-BCL2 as well as N2-M-Bcl-2 (possessing the murine Bcl-2 cDNA) upon NIH 3T3 cells. Consistent with other reports (17, 31), we noted no transformed foci or consistent morphologic changes in the classic NIH 3T3 fibroblast system. Moreover, no differences in colony formation were noted between N2- and N2-H-BCL2-infected NIH 3T3 cells embedded in soft agar (data not shown). However, Reed *et al.* (31) noted that a subpopulation of *BCL2*-transfected 3T3 cells would produce oligoclonal tumors in nude mice, suggesting that in some experimental settings *BCL2* can exert effects in other lineages. Vaux *et al.* (17) noted that *BCL2* conveyed a short-term death-sparing capacity to interleukin 3-dependent lines deprived of their growth factor. The ability of *BCL2* to increase the clonogenicity of LCLs and to complement the tumorigenesis of *MYC*-transfected LCLs may also reflect a *BCL2*-induced survival advantage.

The t(14;18) follicular lymphomas are often regarded as indolent because relatively nonthreatening lymphadenopathy can persist for years before progressing to more aggressive lymphomas after the acquisition of additional genetic changes. *MYC* can be one of these factors, as shown in some cases (26–29) and demonstrated here, but others are likely to exist. The consistent yet subtle effect of *BCL2* upon human B-cell growth and neoplasia is compatible with the disease from which the gene was isolated.

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