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Differential effects of Bcl-2 on T and B cells in transgenic mice

(genetics of lymphoma/apoptosis/T-cell development/T-cell subsets/oncogene)

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ABSTRACT **We have produced *bcl-2* transgenic mice by using a construct which mimics the t(14;18) translocation in human follicular lymphomas. Although lymphoid tissues from all transgenic mice contained high levels of human Bcl-2 protein, transgene expression was differentially regulated within the B- and T-cell compartments of lines derived from various founder mice. We have characterized the phenotypes of two lines of *bcl-2* transgenic mice (line 2 and line 6) in which *bcl-2* transgene expression was restricted primarily to the T- or B-cell lineages, respectively. Analysis of line 6 lymphocytes revealed a polyclonal expansion of B cells, and these B cells exhibited prolonged survival *in vitro*. In line 2 mice, numbers of T cells in the peripheral lymphoid tissues were more moderately elevated despite enhanced T-cell survival *in vitro*. Line 2 transgenic mice also showed significantly increased proportions of thymocytes with a mature phenotype. Taken together, these findings suggest different roles for *bcl-2* in the *in vivo* regulation of B- and T-cell development and homeostasis.**

The t(14;18)(q32;q21) chromosomal translocation is one of the most common cytogenetic abnormalities in lymphoid malignancies, and it occurs in the majority of non-Hodgkin B-cell lymphomas (1, 2). This translocation juxtaposes the *BCL2* protooncogene at chromosome 18q21 with the immunoglobulin heavy-chain (*IGH*) locus at 14q32, resulting in abnormally high levels of *BCL2* gene transcription, probably due to the influence of an enhancer located within the *IGH J-C* intron. The 26-kDa product of the *BCL2* gene has been reported to be localized to the inner mitochondrial membrane (3), but it may also bind to other, as-yet-undefined, structures in a cell-cycle-dependent manner. Bcl-2 is unique among oncogene products in that it appears to enhance lymphoid cell survival by interfering with programmed cell death (also known as apoptosis), rather than promoting cell proliferation (4–7). Though originally discovered because of its involvement in B-cell lymphomas, the *BCL2* gene is normally expressed in both mature B and T cells. As a first step toward defining the *in vivo* functions of *BCL2* in B and T cells, we created several lines of transgenic mice containing a construct that resembles the t(14;18) translocation. Here we describe the effects of *bcl-2* transgene expression on T- and B-cell homeostasis and survival in two strains of *bcl-2* transgenic mice which display T- or B-cell-restricted transgene expression.

MATERIALS AND METHODS

Construction of Human *BCL2/IGH* Minilocus. A DNA construct for microinjection was subcloned from human genomic sequences of *BCL2* and the *IGH* loci (8–10) (Fig. 1). For production of transgenic mice, a 13-kb DNA fragment was isolated from the final plasmid by *Bss*HII digestion, gel-purified, and dialyzed against modified TE buffer (0.1 mM EDTA/10 mM Tris·HCl, pH 7.5).

Production of Transgenic Mice. Approximately 250–500 copies of the construct were microinjected into the male pronucleus of (SWR/J × SJL/J)F_1 fertilized eggs by standard methods (11). Integration of the transgene was initially screened by PCR analysis of tail lysates, using PCR primers specific for the t(14;18) major breakpoint region (12). Results were confirmed by Southern blot analysis of liver DNA isolated from F_1 progeny of each transgenic line. All backcrosses were with SWR/J mice.

Western Blot Analysis. Relative levels of p26-Bcl-2 protein were measured in transgenic tissues and cells by a two-step immunoprecipitation/immunoblot assay that utilizes antibodies specific for the human Bcl-2 (hBcl-2) protein (13).

Flow Cytometry and Cell Purification. Staining and analysis of fresh and cultured lymphocyte populations were performed as previously described (14). Enrichment of T cells was accomplished by incubating cell suspensions with a combination of monoclonal antibodies (mAbs) specific for heat-stable antigen (J11d), the B-cell-specific isoform of CD45, B220 (RA3-3A1/6.1), and major histocompatibility complex I-A antigen (M5/114) for 20 min at 4°C followed by incubation at 37°C for 45 min with 1:10 diluted rabbit complement (Pel-Freez Biologicals, Brown Deer, WI) and centrifugation over Lympholyte M (Cedarlane Laboratories, Hornby, ON, Canada). Enrichment for B cells was performed identically except that mAb 30H12 directed against Thy-1 antigen was used for depletion. Cell populations enriched for B or T cells were >90% pure as measured by flow cytometric analysis.

Immunohistochemistry. Tissue samples for sectioning were fixed in Bouin's solution for 1–2 hr, then processed for paraffin embedding. Serial 5-μm tissue sections were counterstained with hematoxylin for histopathological observations. The pattern of hBcl-2 protein production in lymphoid organs was immunohistochemically examined by using the avidin-biotin-peroxidase complex (ABC) technique with a rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 61-76 of hBcl-2 protein (12, 13).

***In Vitro* Survival.** Primary cell cultures were established at 2×10^6 cells per ml in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 2 mM glutamine, penicillin at 50 units/ml, and streptomycin at 100 μg/ml in a humidified atmosphere of 5% CO_2/95% air at 37°C. Viable

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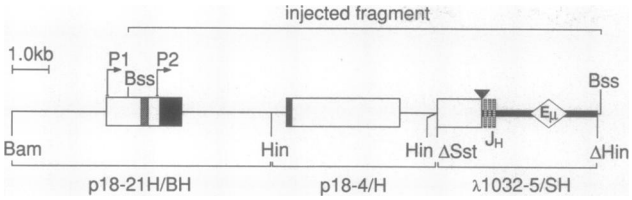

FIG. 1. DNA construct microinjected for production of transgenic mice. A 4.3-kilobase (kb) fragment (λ1032-5/SH) containing a portion of the human *bcl-2* (*BCL2*) 3′ untranslated region, t(14;18) breakpoint, heavy chain joining region (J_H) gene segments, and the *IGH* enhancer (E_μ) region was excised from the λ phage clone λ1032-5 (8) by digestion with *Sst* I and *Hin*dIII, blunted with the Klenow fragment and T4 DNA polymerases, and subcloned in the *Hin*cII site of pSKII (Stratagene). A 6.9-kb fragment (p18-21H/BH) containing two promoters (P1 and P2), the first two exons, and a portion of the second intron of *BCL2* was then excised from p18-21H (10) by *Hin*dIII and partial *Bam*HI digestion, and subcloned in the pSKII/E_μ plasmid. Finally, a 4.4-kb *Hin*dIII fragment (p18-4/H) containing a portion of the second intron, third exon, 3′ untranslated region, and polyadenylylation site of *BCL2* was isolated from p18-4 [probe A (9)] and subcloned in the *Hin*dIII site located between the 5′ *BCL2* and E_μ fragments described above. Endonuclease sites: Hin, *Hin*dIII; Sst, *Sst* I; Bam, *Bam*HI; Bss, *Bss*HII; Δ, deleted sites. Only restriction sites used for subcloning are shown. ▼, t(14;18) breakpoint; ▮, *BCL2* coding region; □, *BCL2* untranslated region; ▨, facultative intron within the first two exons. The thin and thick lines represent chromosome 18 and 14 sequences, respectively.

cells were enumerated in a hemocytometer based on their ability to exclude trypan blue dye.

RESULTS

Transgene Expression in Two Lines of *bcl-2* Transgenic Mice. From seven founder mice, we established three lines of *bcl-2* transgenic mice, two of which (line 2 and line 6) were characterized in detail. An immunoblot assay that utilizes antibodies specific for the hBcl-2 protein was used to determine the tissues in which the *bcl-2* transgene was expressed. The expected p26-Bcl-2 protein was present in thymus, spleen, and lymph nodes derived from both lines of mice but not in liver, kidney, skeletal and cardiac muscle, and brain. Low levels of hBcl-2 protein observed in lung were attributed to infiltrating lymphocytes (Fig. 2 and data not shown). The relative levels of hBcl-2 protein in lymphoid tissues varied among transgenic lines, presumably because of the influence of the integration site on *BCL2/IGH* transgene expression. The hierarchy of hBcl-2 protein expression in line 6 mice was spleen > lymph nodes > thymus, whereas line 2 mice generally had thymus > lymph nodes > spleen. In high-expressing tissues, the levels of transgenic hBcl-2 protein were equivalent to those found in lymphoma cell lines that contain a t(14;18) (Fig. 2).

Enrichment for B cells from spleen and for T cells from lymph nodes showed that the *bcl-2* transgene was expressed predominantly in the B-cell lineage in line 6 mice and in the T-cell lineage in line 2 mice (Fig. 3). These immunoblot data were consistent with immunohistochemical analyses of tissue sections derived from *bcl-2* transgenic mice. For example, the follicular (B-cell) areas of spleens and lymph nodes from line 6 mice exhibited intense immunostaining with anti-hBcl-2 antibodies and these same regions were markedly expanded relative to normal littermate controls (Fig. 4 *A* and *B*). In contrast, in line 2 transgenic mice the T-cell-rich interfollicular areas of spleens and lymph nodes (Fig. 4 *C* and *D*) and the cortex and medulla of thymus sections (15) stained with the anti-hBcl-2 antibodies.

Lymphocyte Populations in Line 2 and Line 6 Transgenic Mice. On gross examination, line 6 transgenic mice consistently exhibited lymphadenopathy and splenomegaly, whereas the lymphoid tissues of line 2 transgenic mice were often indistinguishable from those of littermate controls. Flow cytometric analysis of cell suspensions recovered from line 6 transgenic mice revealed increases of mononuclear cell yields in spleens (2.4-fold) and lymph nodes (2.3-fold), relative to age-matched littermate controls. This was accounted for by 3.1- and 4.4-fold increases in the number of mature $sIgM^+$ $sIgD^+$ (s, surface) B cells in spleens and lymph nodes, respectively. In contrast, mononuclear cell yields of line 2 transgenic mice were elevated by 1.6-fold in spleens and 2.7-fold in lymph nodes relative to age-matched littermate controls. These line 2 transgenic mice showed 1.9- and 3.3-fold increases in cells expressing high levels of the αβ T-cell receptor (TCR^{hi}) in spleens and lymph nodes, respectively. All these increases were statistically significant ($P < 0.05$). However, there were no significant expansions of T cells in line 6 transgenic mice or of B cells in line 2 (Fig. 5).

Although total cell yields were not significantly different from the thymi of line 2 transgenic mice, the percentage of TCR^{hi} thymocytes (48% vs. 32%, $P < 0.005$) was significantly increased in the transgenic thymi, as measured by comparison of a group of 10 line 2 transgenic mice 1–9 months of age with littermate controls by using an unpaired one-tailed *t* test. This was partially accounted for by greater numbers of $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ cells and a corresponding decrease in

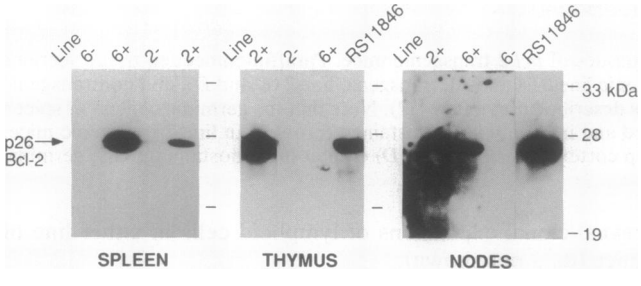

FIG. 2. Lymphoid tissue specific expression of hBcl-2 protein. Protein concentration was normalized to 1 mg/ml, then 500 μl of lysate was used for immunoprecipitations with a rabbit polyclonal antibody against a synthetic peptide corresponding to amino acids 41–54 of hBcl-2 protein. For immunoblots, immunoprecipitates were subjected to SDS/PAGE and transferred to nitrocellulose filters for incubation with a second antiserum specific for amino acids 61–76 of hBcl-2 protein followed by ^{125}I-labeled staphylococcal protein A (13). Representative results are shown for samples from transgenic (+) and nontransgenic (−) littermates of the lines 2 and 6 mice. RS11846 is a human lymphoma cell line that contains a t(14;18).

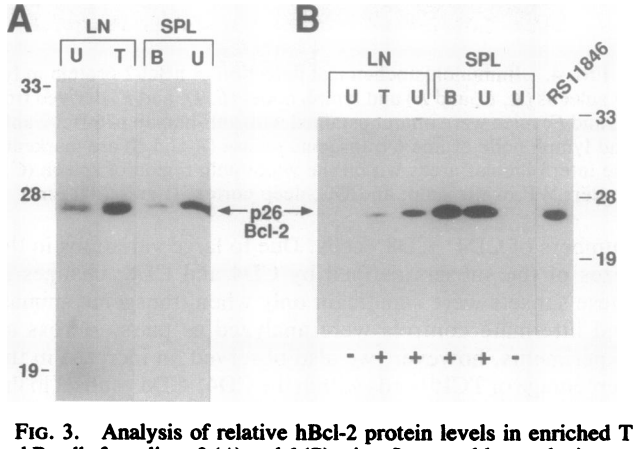

FIG. 3. Analysis of relative hBcl-2 protein levels in enriched T and B cells from lines 2 (*A*) and 6 (*B*) mice. Immunoblot analysis was performed as described previously (see Fig. 2 and ref. 13). U, unfractionated cells; T, enriched T cells; B, enriched B cells; LN, lymph node; SPL, spleen. Scales are in kDa. Representative results are shown for transgenic (+) and nontransgenic (−) littermates.

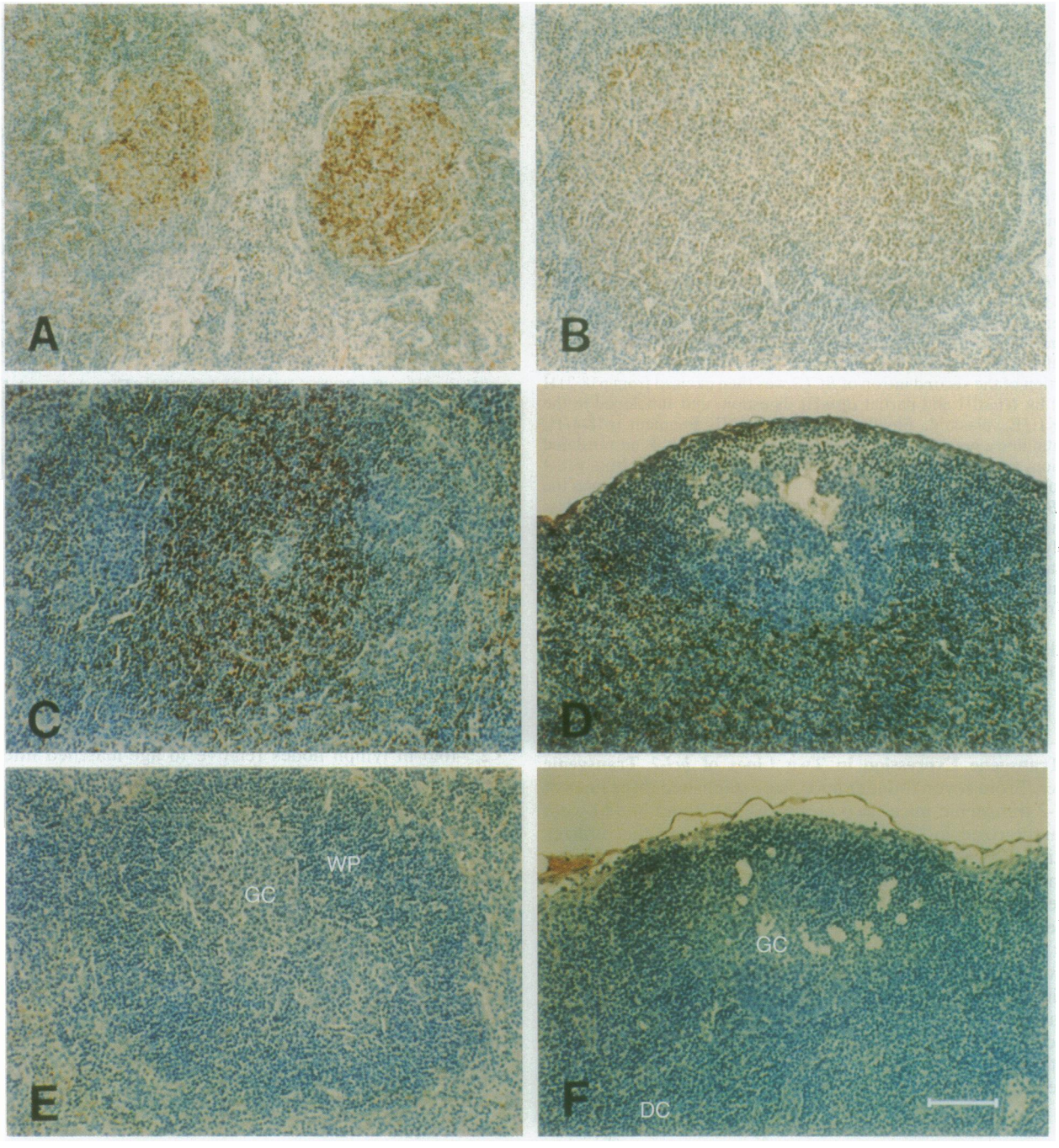

FIG. 4. Immunohistochemical detection of hBcl-2 protein in lymphoid tissues of *bcl-2* transgenic mice. Paraffin-embedded tissue sections of spleens (*A*, *C*, and *E*) and lymph nodes (*B*, *D*, and *F*) derived from transgenic line 6 (*A* and *B*), transgenic line 2 (*C* and *D*), and nontransgenic (*E* and *F*) mice were immunostained with anti-human p26-Bcl-2 antiserum as described previously (12). Note that the germinal centers of spleen and lymph node of line 6 transgenic mouse (*A* and *B*) are markedly enlarged and intensely immunostained (brown). In line 2 transgenic mice, the interfollicular areas within the white pulp region of spleen (*C*) and deep cortex of lymph node (*D*) exhibit immunostaining. GC, germinal center; WP, white pulp; and DC, deep cortex. (Bar = 100 μm.)

numbers of $CD4^+$ $CD8^+$ cells. Due to large variations in the sizes of the subsets defined by CD4 and CD8, changes in these subsets were significant only when transgenic animals and littermate controls were analyzed as pairs. Across all experiments, however, we also observed an increase in the percentage of TCR^{hi} cells within the $CD4^+$ $CD8^+$ subset in the transgenic animals (18% vs. 9%, $P < 0.01$). Despite these population abnormalities, DNA content analysis and thymidine incorporation assays revealed no increased proliferation in thymic or peripheral lymphocytes from either transgenic strain. In addition, Southern blot analysis using multiple immunoglobulin and T-cell receptor gene probes also failed to reveal clonal expansions of lymphoid cells in either line of mice (data not shown).

Prolonged Survival of Transgenic Lymphoid Cells in Culture. *In vitro* comparisons of lymphocytes recovered from line 6 transgenic mice with their nontransgenic littermates demonstrated markedly prolonged survival of splenic and lymph node B cells after 10 days (Fig. 6 and data not shown). A clear enhancement of the survival of spleen- and lymph node-derived lymphocytes was also noted in line 2 mice, but in this case T cells rather than B cells made up the majority of viable cells after 8 days of culture (Fig. 6 and data not shown). Thymocytes from younger line 2 mice whose ratios

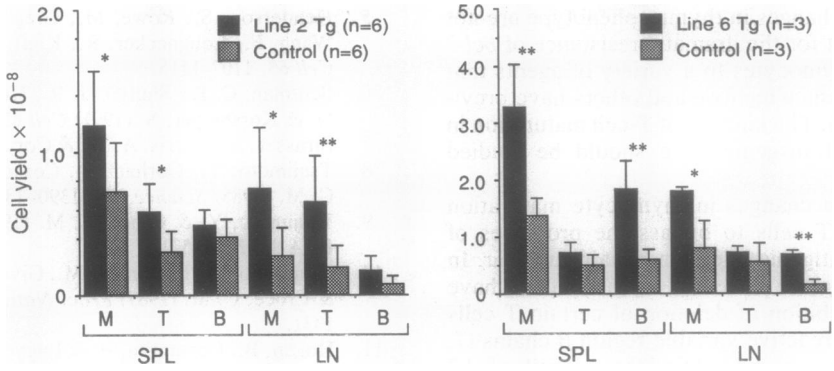

FIG. 5. Analysis of lymphocyte subpopulations in *bcl-2* transgenic mice. Mononuclear cells (M) were recovered from spleens (SPL) and lymph nodes (LN) of age-matched pairs of transgenic and nontransgenic littermates. The numbers of T and B cells were calculated by multiplying the total mononuclear cell yield by the proportion of T or B cells as determined by flow cytometry. Statistical analysis was accomplished with an unpaired one-tailed *t* test using STATVIEW SE software (Abacus Concepts, Berkeley, CA). Data represent mean cell yields for transgenic (Tg) mice (black bars) and age-matched nontransgenic littermates (stippled bars). The error bars depict one standard deviation from the mean. * and ** represent statistical significance at the levels of $P < 0.05$ and $P < 0.01$, respectively.

of mature to immature cells were comparable to those of control mice also exhibited prolonged survival *in vitro*, with 7–10 times more cells viable after 7 days of culture. The surviving cells in these thymocyte cultures consisted of both phenotypically immature and mature phenotypes (data not shown).

DISCUSSION

The data presented here extend previous findings regarding the *in vivo* effects of *bcl-2* overexpression in transgenic mice (6, 7, 16). In the line 6 mice, where hBcl-2 protein production appeared to be limited predominantly to the B-cell lineage, polyclonal expansion of B cells was noted. This confirms the ability of deregulated hBcl-2 expression to provide a selective survival advantage to B cells *in vivo*. Though T cells derived from the line 2 mice expressed the *BCL2/IGH* transgene at levels comparable to those found in t(14;18)-containing lymphoma cell lines and exhibited extended survival in culture similar to the extended B-cell survival found in line 6 mice, *in vivo* expansion of peripheral T cells was only approximately 60% of that seen in B cells. However, the modest expansion of the T-cell pool reported here is unlike that in other transgenic mice expressing *bcl-2* in peripheral T cells, where no increases in peripheral T-cell counts were found (7).

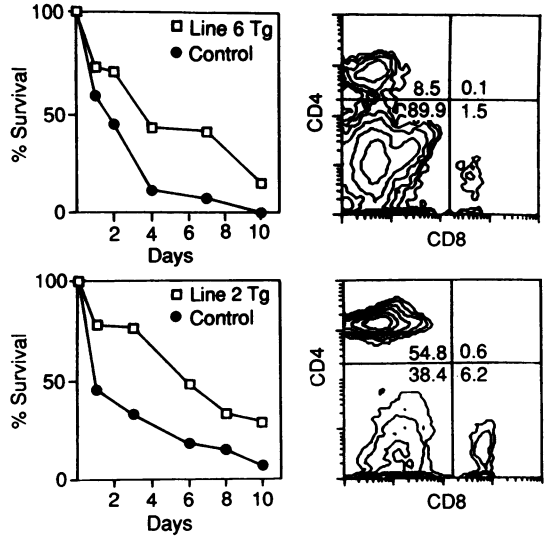

FIG. 6. Extended *in vitro* survival of transgenic splenocytes. Mononuclear cells were enriched from spleens by ammonium chloride lysis of erythrocytes and cultured at 2×10^6 cells per ml in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. (*Left*) At various times thereafter, numbers of surviving cells were determined by trypan blue dye exclusion. (*Right*) Two-color flow cytometry (phycoerythrin for CD4; fluorescein isothiocyanate for CD8) was performed on the surviving cells after 10 days (line 6; *Upper*) or 8 days (line 2; *Lower*) of culture *in vitro*. The proportion of surviving surface immunoglobulin (sIg)$^+$ cells was 76% for the line 6 transgenic (Tg) mouse and 30% for the line 2 mouse (data not shown). Starting immunophenotype for the line 6 mouse was 18% CD4$^+$ CD8$^-$, 6% CD4$^-$ CD8$^+$, 75% CD4$^-$ CD8$^-$, 48% sIg$^+$, and 1% CD4$^+$ CD8$^+$. For the line 2 mouse, it was 25% CD4$^+$ CD8$^-$, 6% CD4$^-$ CD8$^+$, 68% CD4$^-$ CD8$^-$, and 1% CD4$^+$ CD8$^+$.

The differential *in vivo* effects of high-level Bcl-2 protein expression on B and T cells may result from differences in the normal life-spans of these cell types. T cells are thought to have half-lives of months to years *in vivo*, as opposed to B cells, which have been estimated to live only 5–7 days unless stimulated to enter a memory-type pathway (17, 18). Thus, deregulated Bcl-2 expression may have less of an influence on mature T cells than B cells because the former are intrinsically long-lived. Alternatively, differences in the way that expression of the *bcl-2* gene is normally regulated in B and T cells may contribute to the different phenotypes *in vivo*. Circulating peripheral blood T cells, for instance, normally contain substantial levels of p26-Bcl-2 protein which do not change appreciably when they are stimulated to proliferate (19). In contrast, levels of Bcl-2 protein appear to decline as circulating B cells enter the germinal centers of nodes and begin to proliferate in response to antigens, thus creating a period of vulnerability to apoptotic death (20). The increased influence of deregulated *bcl-2* expression on B cells *in vivo* may explain why human malignancies involving Bcl-2 overexpression have been described thus far only for B cells, not for T cells.

Though mature peripheral T cells are long-lived, this is clearly not the case for immature thymocytes, which have been estimated to have an average half-life of 2–3 days (21). Although numbers of thymocytes in line 2 mice were not significantly elevated, we did observe an increase in the proportions of thymocytes expressing mature surface phenotypes. When a large group of mice were examined one of the most striking differences between transgenic mice and littermate controls was a shift toward cells expressing higher levels of the T-cell receptor in the normally immature CD4$^+$ CD8$^+$ compartment. TCR^{hi} CD4$^+$ CD8$^+$ cells have been found to be postselection intermediates in the T-cell developmental pathway and have been found to be the immediate precursors of CD4$^-$ CD8$^+$ or CD4$^+$ CD8$^-$ mature T cells (21, 22). Our observations thus imply that altered kinetics or efficiency of repertoire selection may be occurring in these

mice. However, these changes in thymus phenotype are not great enough to account for the dramatic resistance of *bcl-2* transgenic immature thymocytes to a variety of agents that normally induce apoptosis which we and others have previously observed (6, 7, 15). The kinetics of T-cell maturation in the thymus of line 2 transgenic mice should be studied further.

Whether or not these changes in thymocyte maturation allow *bcl-2* transgenic T cells to bypass the processes of positive and negative antigenic selection remains unclear. In models of negative antigenic selection, we and others have observed a modest inhibition of deletion of certain T cells bearing potentially autoreactive variable region β chains (7, 15). Another group, however, did not find this in similar *bcl-2* transgenic mice (6). These findings imply that other mechanisms for mediating immune tolerance remain intact despite the effects of Bcl-2 expression in the B- or T-cell compartments. The question of whether *bcl-2* overexpression will allow thymocytes to bypass positive selection requires further study. For these and other issues, future investigations of these transgenic mice are likely to provide useful insights into the mechanisms of immune cell development and homeostasis and the role of *bcl-2* in these processes.

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