

Inhibition of thymocyte apoptosis and negative antigenic selection in *bcl-2* transgenic mice

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ABSTRACT The *bcl-2* gene, which is overexpressed in human follicular B-cell lymphomas, has been found to extend cellular lifespan through inhibition of apoptosis, or programmed cell death. However, the physiological role of the Bcl-2 protein in lymphocyte development is unclear. We have established a transgenic mouse line that expresses high levels of the Bcl-2 protein in both cortical and medullary thymocytes, disrupting the normal pattern of expression of this gene. We found that in these mice, immature thymocytes became resistant to apoptosis mediated by corticosteroids and calcium ionophores. Untreated thymocytes also exhibited a survival advantage in suspension cultures compared with controls. In addition, overexpression of *bcl-2* enabled a proportion of thymocytes and peripheral T cells to escape the process of clonal deletion, which normally eliminates self-reactive T cells during thymocyte maturation. These findings implicate the Bcl-2 protein in regulating the lifespan of maturing thymocytes and in the antigenic-selection process.

In most human follicular lymphomas, the *BCL2* gene is overexpressed as a result of chromosomal translocation into the immunoglobulin heavy chain locus (1–3). The p26 Bcl-2 protein is expressed in intracellular membranes, particularly within mitochondria (4). Overexpression of *bcl-2* in cell lines has been found to extend cellular survival without inducing proliferation by inhibiting programmed cell death, also termed apoptosis (4, 5). During T-cell development, thymocytes mature from short-lived, steroid-sensitive CD4⁺CD8⁺ cells into very long-lived, steroid-resistant CD4⁺CD8⁺ or CD4⁺CD8⁻ mature T cells. CD4⁺CD8⁺ thymocytes that are not selected to mature rapidly disappear from the thymus, probably as a result of programmed cell death (6–8). Although *bcl-2* expression is normally restricted to the mature T cells in the thymic medulla that have survived thymic selection (9, 10), it is not known whether this pattern of expression contributes to the lifespan changes during thymocyte development. To better understand the contribution of *bcl-2* to T-cell development, we studied the phenotype of transgenic mice expressing high levels of the human Bcl-2 protein in thymocytes and peripheral T cells.

MATERIALS AND METHODS

Transgenic Mice. The transgene construct contained portions of the human *BCL2* gene and a t(14;18) breakpoint region with the immunoglobulin heavy chain enhancer. (SWR × SJL) F₁ transgenic mice were generated as described (11) and propagated on an SWR background. Transgenic status was routinely determined by PCR analysis of tail lysates using *bcl-2*- and joining region of the heavy chain-specific primers (12). The expression of the *bcl-2* transgene was

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monitored by immunoblot and immunohistochemical assays (12, 13).

Induction and Analysis of Thymocyte Apoptosis. Mice were injected i.p. with dexamethasone (Dex) at 50 mg/kg in phosphate-buffered saline, or phosphate-buffered saline alone. Forty-eight hours later, thymocyte cell suspensions were prepared for fluorescence-activated cell sorter (FACS) analysis after incubation with phycoerythrin-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD8 monoclonal antibodies (mAbs) as described (11). For *in vitro* treatments, thymocytes were isolated and cultured at 2 × 10⁶ cells per ml in Dulbecco's modified Eagle's medium/5% fetal calf serum with or without various concentrations of Dex, ionomycin, or cycloheximide (Sigma). Cells were stained with anti-CD4 and anti-CD8 mAbs as above, and propidium iodide at 25 μg/ml was added immediately before FACS analysis. Cells that did not stain with propidium iodide were considered viable. CD4^{dull}CD8^{dull} cells were separated from the rest of the CD4⁺CD8⁺ population because these cells have been shown to be in the early stages of apoptosis (14). DNA fragmentation assays were accomplished by a gel-electrophoresis method as described (15).

T-Cell β Chain of the Variable Region (Vβ) Use. Transgenic mice were back-crossed to the AKR/J strain (Mls-1^a, I-E^k). Transgenic and control offspring from 4 to 20 weeks were sacrificed, and suspensions of thymus, spleen, lymph node, and peripheral blood lymphocytes were analyzed by FACS with biotinylated anti-Vβ17a (mAb KJ23a, Pharmingen, San Diego) followed by streptavidin-Red613, or using anti-Vβ6 (mAb RR4-6, from J. Punt, University of Pennsylvania) followed by fluorescein isothiocyanate-conjugated mouse anti-rat immunoglobulin serum (Jackson ImmunoResearch). For anti-Vβ6 staining, rat IgG at 0.5 mg/ml was used in subsequent stainings to block crossreactivity of the anti-rat reagent. Appropriately conjugated anti-CD4 and anti-CD8 mAbs (Becton Dickinson and GIBCO) were used in conjunction with anti-Vβ antibodies and biotinylated anti-T-cell receptor (mAb H-57-597, Pharmingen) for three-color staining to identify total percentages of T cells.

RESULTS

T-Cell Expression in a Line of *bcl-2* Transgenic Mice. We have generated a number of transgenic mouse lines with a construct containing the human proximal *BCL2* promoter, *BCL2*-coding sequences, and the human immunoglobulin heavy chain joining-constant region with its accompanying

Abbreviations: Dex, dexamethasone; FACS, fluorescence-activated cell sorter; Vβ, β chain of variable region; mAb, monoclonal antibody. *To whom correspondence specifically regarding the transgenic mice should be addressed at: Room 252, John Morgan Building, University of Pennsylvania School of Medicine, 36th Street and Hamilton Walk, Philadelphia, PA 19104.

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transcriptional enhancer. In mice derived from one founder (no. 6), we observed follicular B-cell accumulation and extended survival of resting B cells, as has been reported (16, 17). However, in mice derived from another founder (no. 2), we found a strikingly different phenotype, with an absence of follicular hyperplasia and an accumulation of mature thymocytes in the thymi of older mice (M.K., R.M.S., D.C.L., T.M., Y. Tsujimoto, P. C. Nowell, M.I.G., and J.C.R., unpublished work). Immunoblot analysis with antiserum specific for the human Bcl-2 protein showed that the highest levels of the transgenic Bcl-2 protein accumulation occurred in the thymus of line 2 *bcl-2* transgenic mice, with slightly less in the lymph nodes and relatively little in spleen (Fig. 1A). Lymphocyte fractionation and immunohistochemical studies showed that most transgene expression in this line occurs in T rather than in B cells (unpublished data). Levels of Bcl-2 expression in the thymus were comparable to those in human follicular lymphoma cell lines and considerably above those found in normal tissues expressing endogenous *bcl-2*. When thymus tissue sections from line 2 *bcl-2* transgenic mice were immunohistochemically evaluated by using an antiserum specific for the human Bcl-2 protein (13), we observed intense immunostaining in the thymic cortex, where *bcl-2*-expressing cells are normally rare (9, 10) (Fig. 1B). Expression of transgenes linked to the IgH enhancer in T cells is not unusual and is thought to depend on the promoters included in the construct as well as the chromosomal integration site of the transgene (18, 19). Moreover, transgene expression driven by the IgH enhancer has been found in all four thymocyte subsets defined by CD4 and CD8 expression (20).

Effect of *bcl-2* Overexpression on Thymocyte Apoptosis. Because normal cortical CD4⁺CD8⁺ double-positive thymocytes usually have a lifespan of ≈ 3 –4 days and are readily induced to undergo apoptotic cell death after steroid exposure (8, 21), we sought to determine the effects of *bcl-2* expression on these cells. Four-to-twelve-week-old line 2 *bcl-2* transgenic mice and age-matched nontransgenic littermates were treated with Dex at 50 mg/kg, and their thymus glands were examined 48 hr later (Fig. 2). Greater numbers of thymocytes were consistently recovered from Dex-treated transgenic mice compared with controls. The increased yields could be accounted for primarily by increased survival of normally steroid-sensitive CD4⁺CD8⁺ cells. In the experiment shown in Fig. 2A, nine times more CD4⁺CD8⁺ cells (4.4×10^6) were recovered from Dex-treated transgenic thymi compared with controls (4.9×10^5). Similarly increased numbers of Dex-resistant thymocytes with a T-cell receptor TCR^{dull} phenotype (3.1×10^6 vs. 3.8×10^5) were recovered from transgenic animals, consistent with enhanced survival of immature cells (22).

To examine the effects of *bcl-2* expression on immature thymocytes in more detail, we treated thymocytes from young adult transgenic mice with various concentrations of Dex in suspension cultures and determined the phenotype of surviving cells by flow cytometry (Fig. 3A). Transgenic thymocytes displayed marked resistance to steroid-mediated cell death *in vitro*, with increased survival of immature transgenic CD4⁺CD8⁺ cells again accounting for most of the difference in total viable cell yields. Consistent with their enhanced survival, Dex-treated *bcl-2* transgenic thymocytes were found to undergo markedly less DNA fragmentation than controls (Fig. 3B). The mechanism of thymocyte death in these experiments was consistent with previous reports of apoptosis in that it was accompanied by DNA digestion into oligonucleosomal fragments and was preventable by the protein-synthesis inhibitor cycloheximide (23). Furthermore, the DNA fragmentation could be detected as early as 4 hr after Dex treatment, well before loss of cellular viability was detected by trypan blue or propidium iodide uptake. Because Dex-mediated cell death is thought to require a prolonged

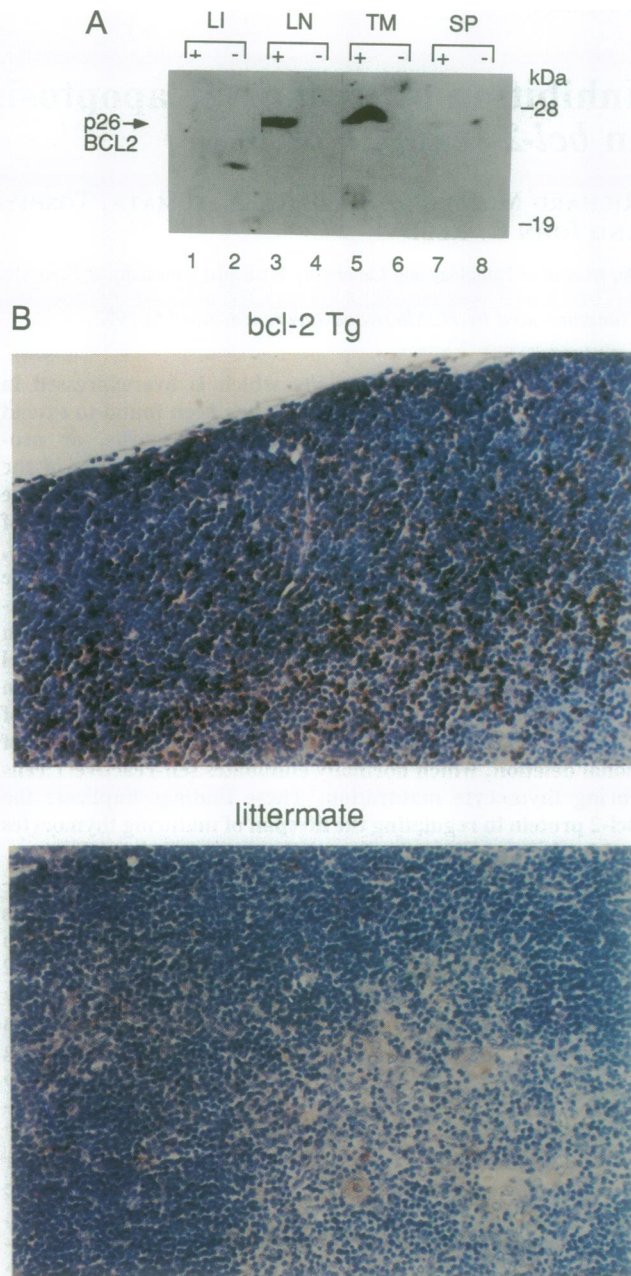


FIG. 1. Pattern of transgene expression in line 2 *bcl-2* transgenic mice. (A) Immunoblot analysis of liver (LI), lymph node (LN), thymus (TM), and spleen (SP) from line 2 *bcl-2* transgenic mice (+) and normal littermates (-). Levels of p26 Bcl-2 found in transgenic spleen varied among individual animals but always were less than those in lymph node or thymus. (B) Immunohistochemical analysis of thymus sections from a 4-week-old *bcl-2* transgenic mouse (Upper) and normal littermate (Lower). Scattered strongly Bcl-2-positive cells (brown) can be seen throughout the transgenic (Tg) thymus, with particularly strong staining at the junction between the densely packed thymic cortex and the more hypocellular thymic medulla. There was no significant immunostaining for endogenous Bcl-2 in the nontransgenic littermate thymus, consistent with previous demonstration of the specificity of this antiserum for the human form of the Bcl-2 protein (13). ($\times 200$.)

increase in intracellular Ca²⁺ (23), we used the calcium ionophore ionomycin to determine whether *bcl-2* acts proximally or distally to the effects of calcium influx. *bcl-2* expression markedly inhibited the induction of apoptosis by ionomycin (Fig. 3C). When cultured for extended periods in the absence of Dex or ionomycin, transgenic thymocytes also exhibited a survival advantage, with seven to ten times more

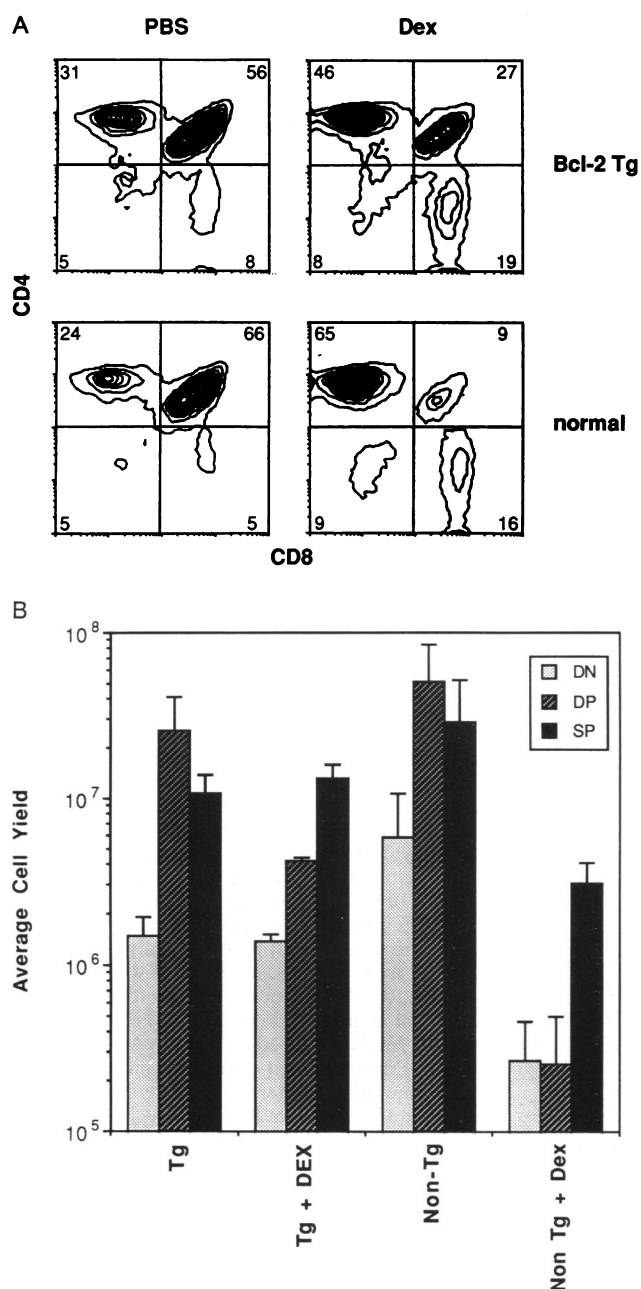


FIG. 2. Immunophenotypic analysis of thymocytes recovered from Dex-treated *bcl-2* transgenic (Tg) mice. (A) Two-color contour plots from FACS analysis of CD4 and CD8 surface expression are shown for thymocytes from animals sacrificed 48 hr after Dex or vehicle (phosphate-buffered saline; PBS) injection. Percentages in each quadrant are indicated by the numbers shown. (B) Mean cell yields of double-negative (CD4⁻CD8⁻; □), double-positive (CD4⁺CD8⁺; ⊗), and single-positive (CD4⁺CD8⁻ or CD4⁻CD8⁺; ■) thymocytes after *in vivo* Dex treatment were determined from two independent experiments. Yields were obtained by multiplying the percentages obtained by FACS analysis by the total number of cells recovered from each thymus. Error bars represent 1 SD from means.

cells remaining viable relative to controls after 1 week (data not shown).

Negative Selection by Self-Antigens in *bcl-2* Transgenic Mice. The increased survival of *bcl-2* transgenic thymocytes observed after treatment with pharmacological inducers of programmed cell death suggested that *bcl-2* expression might confer resistance to the T-cell-specific physiological process of clonal deletion, which is thought to involve apoptosis of self-reactive cortical CD4⁺CD8⁺ thymocytes (7, 24, 25). To

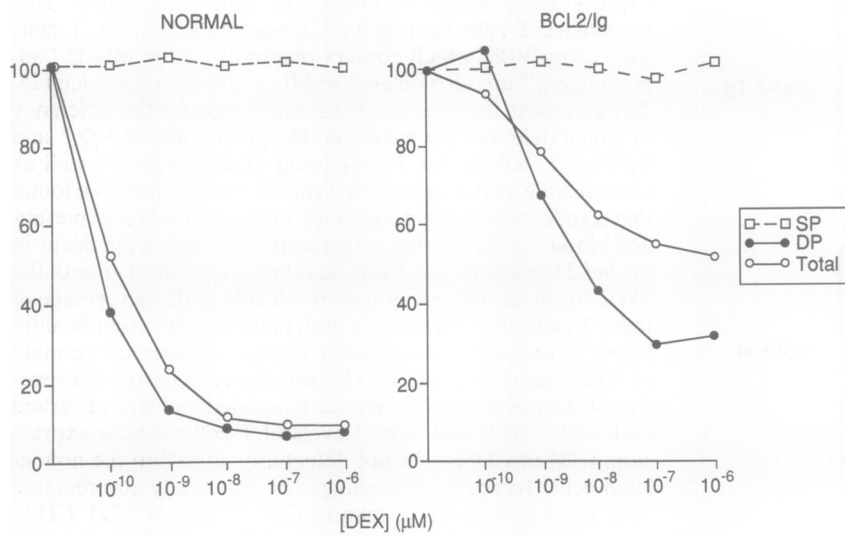
determine whether this is the case, we mated the line 2 *bcl-2* transgenic mice with mice of the AKR strain. AKR mice express I-E^k, which is known to efficiently delete I-E-responsive T cells bearing Vβ17a segments (26, 27). T cells expressing Vβ6, which confers reactivity to the Mls-1^a "superantigen," are also deleted in Mls-1^a, H-2^k AKR mice (25, 28). To assess the effect of *bcl-2* expression on the efficiency of clonal deletion, we measured the percentage of Vβ6⁺ and Vβ17a⁺ T cells in the F₁ offspring of this cross as well as control AKR (deleting) or SWR (nondeleting) mice. We found that a reduction in the percentage of lymph node and peripheral blood T cells bearing autoreactive Vβ chains did occur in the *bcl-2* transgenic × AKR mice relative to control mice of the SWR strain. However, as shown in Table 1, the percentage of these T cells was consistently and, in most cases, significantly higher in transgenic mice than in the nontransgenic littermate or AKR controls (Table 1). This difference occurred irrespective of the age or sex of the mice examined and was observed both with Vβ17a and Vβ6. Levels of T-cell receptor expression, CD4 or CD8, were not detectably altered in the nondeleted cells. Increased percentages of potentially autoreactive cells were also found in mature CD4⁺CD8⁻ or CD4⁻CD8⁺ thymocytes, indicating that the partial defect in clonal deletion of Vβ17a- and Vβ6-bearing T cells in the *bcl-2* transgenic mice probably occurs during thymic development. To show that these effects were specific, we measured the percentage of T cells expressing Vβ8.1 and Vβ8.2, by using the mAb KJ16. Most mAb KJ16⁺ T cells in these strains express Vβ8.2, which is not affected by I-E or Mls-1 self-antigens (28). The percentage of mAb KJ16⁺ T cells did not differ significantly between the transgenic mice and nontransgenic littermate or AKR controls (data not shown).

DISCUSSION

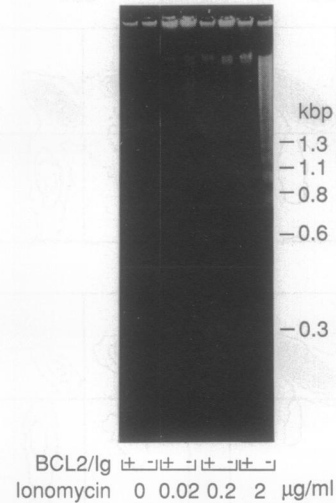
Recent studies have shown that thymocytes that lack minimal reactivity with self-major histocompatibility complexes or those with high affinity for self-antigens are eliminated from the T-cell repertoire in the thymus at the CD4⁺CD8⁺ stage, most likely through programmed cell death (6, 7, 29). Transgene-mediated expression of Bcl-2 in CD4⁺CD8⁺ thymocytes conferred extended survival and resistance to apoptosis on these cells when exposed to Dex or ionomycin. We also observed a partial defect in the elimination of T cells bearing potentially autoreactive T-cell-receptor β chains in these mice. Whether or not the cells that escaped deletion are part of a low-affinity subpopulation with reduced self-reactivity could not be determined due to the small number of cells bearing these receptors. However, we have never observed any evidence of autoimmune disease in these animals at up to 2 yr of age, and in mixed-lymphocyte culture experiments we have not seen any increase in responses to autologous cells by *bcl-2* transgenic lymphocytes. If high-affinity autoreactive T cells do escape deletion, then other mechanisms, such as peripheral induction of clonal anergy may still operate in the *bcl-2* transgenic mice to prevent autoimmunity.

The resistance to negative selection and apoptosis in these *bcl-2* transgenic thymocytes was not absolute. This fact could reflect expression of the transgene in only a percentage of thymocytes, as suggested by the nonconfluent *bcl-2* immunostaining in the thymus (Fig. 1B). Alternatively, because CD4⁺CD8⁺ thymocytes are themselves heterogeneous in their expression and signaling through the T-cell receptor and in their sensitivity to antigenic selection (30–32), *bcl-2* expression may be able to rescue only a subpopulation of these cells from steroid- or antigen-induced apoptosis. Further studies with transgenic mice expressing a single T-cell receptor specificity are necessary to fully elucidate the nature of the defect in clonal deletion seen in these animals. It also remains to be determined whether *bcl-2* transgenic thymo-

A DEXAMETHASONE - MEDIATED KILLING OF THYMOCYTES



C



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BCL2/Ig: + - + - + -

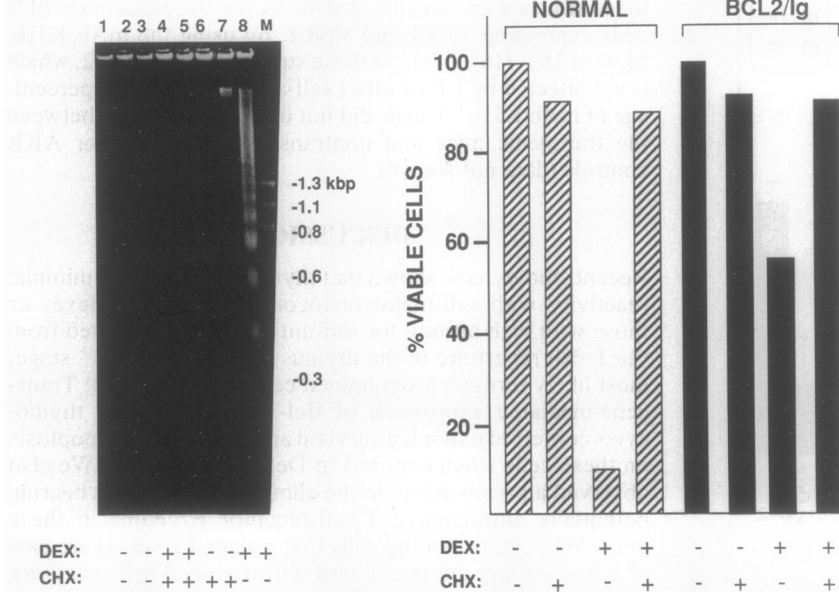


FIG. 3. Resistance of *bcl-2* transgenic thymocytes to apoptosis induced by Dex and ionomycin *in vitro*. (A) Percentages of viable CD4⁺CD8⁻ single positive (SP; □), brightly staining double-positive CD4^{bright}CD8^{bright} (DP; ●), and total (○) thymocytes were determined for cultures of *bcl-2* transgenic (Tg) and normal littermate thymocytes after 18 hr of Dex treatment. Values are normalized to untreated cells (100%). (B) (Left) Photograph shows gel electrophoresis analysis of DNA derived from 10⁶ cells treated for 4 hr with or without 10⁻⁷ M Dex or cycloheximide (CHX) at 15 μg/ml as indicated. Molecular-mass-markers (M) are *Hae*III-digested φX174 DNA fragments. (Right) Data represent cellular viability of *bcl-2* transgenic (■) and littermate (▨) thymocytes determined by propidium iodide exclusion at 18 hr. Values are normalized to untreated cells (100%). (C) Gel electrophoresis analysis showing levels of DNA degradation induced in *bcl-2* transgenic (+) and nontransgenic littermate (-) thymocytes after 4 hr of culture with the indicated ionomycin concentrations. kbp, kilobase pairs.

cytes can bypass the requirements for positive selection of a self-restricted T-cell repertoire.

After completion of these experiments, results with independently generated transgenic mice expressing human

Table 1. Effect of I-E and Mls-1^a expression on the frequency of Vβ6⁺ and Vβ17a⁺ T cells in *bcl-2* transgenic mice

Strain	mIs-1	MHC	I-E	Vβ17a PBLT	Vβ6 PBLT	Vβ17a LNT	Vβ6 LNT	Vβ17a thymus	Vβ6 thymus
AKR*	a	k	+	0.55 ± 0.45	0.66 ± 0.43	1.27 ± 0.24	0.24	1.16 ± 1.04	0.49
SWR†	a	q	-	11.12 ± 1.91	5.87 ± 0.25	11.22 ± 0.77	5.67	12.38 ± 1.19	5.85
AKR × SWR <i>Bcl-2</i> Tg‡	a	k × q	+	2.40 ± 0.24	1.25 ± 0.3	2.33 ± 0.24	1.05 ± 0.11	2.50 ± 0.35	1.77 ± 0.42
AKR × SWR non-Tg‡	a	k × q	+	1.24 ± 0.28	0.47 ± 0.24	1.55 ± 0.22	0.26 ± 0.05	1.24 ± 0.20	0.15 ± 0.13
Significance (Tg vs. non-Tg)				P ≤ 0.04	P ≤ 0.01	P ≤ 0.06	P ≤ 0.02	P ≤ 0.002	P ≤ 0.06

Values are the percentage of each cell type staining for the indicated Vβ, after subtraction of staining with the secondary reagent alone (mean ± SEM for two to six animals per group, or for one animal in some AKR or SWR controls). PBLT, peripheral blood T cells; LNT, lymph node T cells; thymus, CD4⁺CD8⁻ or CD4⁻CD8⁺ thymocytes. MHC, major histocompatibility complex. A two-tailed unpaired Student's *t* test was used to measure the significance of the differences between Vβ frequencies seen in *bcl-2* transgenic (Tg) and nontransgenic (non-Tg) mice on the AKR × SWR background. Data were analyzed by using STATVIEW SE software (Abacus Concepts, Berkeley, CA).

*The Vβ17a gene is not present in AKR mice.

†Vβ6⁺ T cells are not deleted in H-2^s, Mls-1^a strains (25).

‡These mice have one Vβ17a allele, which results in expression of up to one-half the percentage of Vβ17a cells compared with SWR mice, which have two Vβ17a alleles (26).

BCL2 in T cells were reported (33, 34). All of these reports are in agreement with regard to resistance to pharmacologically induced apoptosis and extended *in vitro* survival. However, our findings differ from other reports with regard to negative selection of T cells expressing potentially autoreactive $V\beta$ chains. Although we observed partial escape from negative selection in both the periphery and thymus of our *bcl-2* transgenic mice, Strasser *et al.* (33) found this phenomenon only in the thymus, and Sentman *et al.* (34) found clonal deletion unaffected. A number of reasons may account for these discrepancies. (i) The absence of escape from clonal deletion seen by Sentman *et al.* (34) may relate to the relative levels of transgene expression in mature and immature thymocytes. Although they report expression of the transgene in both the thymic cortex and medulla, expression of the *bcl-2* transgene in their case was driven by the proximal *lck* promoter, which is known to confer greater expression in immature $CD4^+CD8^+$ thymocytes than in mature thymocytes and peripheral T cells (35, 36). Clonal deletion of autoreactive cells has been found to occur after commitment to the $CD4$ or $CD8$ lineages (30), and the *lck* promoter may not provide sufficient expression of *bcl-2* at this critical developmental stage to protect these cells from apoptosis. (ii) Although we can find no obvious explanation for the failure of Strasser *et al.* (33) to observe escape from clonal deletion in the periphery of their *bcl-2*/IgH transgenic mice, subtle differences in the levels of *bcl-2* expression may be present due to the different promoter elements used in their study or the integration site of the transgene. (iii) In addition, we cannot rule out the contribution of the different mouse strain combinations used in all of these studies.

Despite these differences in sensitivity to clonal deletion, all of these reports agree on the identification of *bcl-2* as a gene product that specifically interferes with steroid-mediated apoptosis. The precise mechanism by which the Bcl-2 protein accomplishes this remains enigmatic, but previous studies of steroid-mediated thymocyte apoptosis have implicated increases in cytosolic Ca^{2+} and activation of endogenous endonucleases in this process (23). The observation that *bcl-2* blocks apoptosis induced by ionomycin suggests that *bcl-2* may function to stabilize Ca^{2+} levels or, more likely, act downstream of Ca^{2+} influx in a pathway that provides signals for apoptotic cell death. Our observations may also help explain why human B-cell neoplasms overexpressing *bcl-2* are relatively resistant to chemotherapy regimens that include glucocorticoids (37, 38).

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