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Developmental regulation of the Bcl-2 protein and susceptibility to cell death in B lymphocytes

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Cell death is a prominent feature of B cell development. For example, a large population of B cells dies at the pre-B cell stage presumably due to the failure to express a functional immunoglobulin receptor. In addition, developing B cells expressing antigen receptors for self are selectively eliminated at the immature B cell stage. The molecular signals that control B cell survival are largely unknown. The product of the *bcl-2* proto-oncogene may be involved as its overexpression inhibits apoptotic cell death in a variety of biological systems. However, the physiological role of the endogenous Bcl-2 protein during B cell development is undetermined. Here we show a striking developmental regulation of the Bcl-2 protein in B lymphocytes. Bcl-2 is highly expressed in CD43$^+$ B cell precursors (pro-B cells) and mature B cells but downregulated at the pre-B and immature B cell stages of development. We found that Bcl-2 expressed by B cells is a long-lived protein with a half-life of ~10 h. Importantly, susceptibility to apoptosis mediated by the glucocorticoid hormone dexamethasone is stage-dependent in developing B cells and correlates with the levels of Bcl-2 protein. Furthermore, expression of a *bcl-2* transgene rescued pre-B and immature B cells from dexamethasone-induced cell death, indicating that Bcl-2 can inhibit the apoptotic cell death of progenitors and early B cells. Taken together, these findings argue that Bcl-2 is a physiological signal controlling cell death during B cell development.

Key words: apoptosis/B cell development/Bcl-2/cell death

Introduction

In the adult mouse, $3-5 \times 10^7$ newly formed B cells are generated per day in the bone marrow (BM) by proliferation and differentiation of early B cell progenitors (Osmond, 1990; Osmond *et al.*, 1990). During maturation, pro-B and pre-B cells undergo an ordered sequence of immunoglobulin heavy (IgH) and light (IgL) chain gene rearrangements and the loss or acquisition of several lineage-related molecules (Tonegawa, 1983; Forster *et al.*, 1989; Hardy *et al.*, 1991; Rolink and Melchers, 1991; Ehlich *et al.*, 1993). Successful assembly of functional IgH and IgL chains ultimately leads to expression of IgM antigen receptors on the surface of newly formed B cells. Further maturation of IgM$^+$IgD$^-$ immature B cells results in the co-expression of surface IgM and IgD which defines the mature B cell population. Despite the large number of B cell precursors generated, only $2-3 \times 10^6$ IgM$^+$IgD$^+$ mature B cells enter the blood-stream and are incorporated into the peripheral pool (Forster *et al.*, 1989; Osmond, 1990; Osmond *et al.*, 1990). The extensive loss of B lineage cells during maturation reflects a powerful selection process by which those B cells undergoing productive assembly of IgH and IgL chains are incorporated into the peripheral pool (Rolink and Melchers, 1991). Since the mechanism that mediates IgH and IgL chain gene recombination is imprecise and error prone, a large fraction of the pre-B and immature B cells generated fails to express functional IgM molecules. These non-functional B cell precursors undergo cell death in the BM. Evidence for extensive cellular loss has come from labelling experiments which have showed that up to 75% of the developing B cells are eliminated at the transition from the large pro-B cell stage to the small pre-B cell stage (Opstelten and Osmond, 1983; Osmond *et al.*, 1992; Ehlich *et al.*, 1993).

In addition to the mechanism that ensures the selection of IgM$^+$ B cells, newly formed B cells undergo a second check at the immature IgM$^+$IgD$^-$ B cell stage. This second selection process is based on the specificity of the IgM receptor displayed by the B cell. Immature B cells expressing IgM receptors for self undergo physical elimination (clonal deletion) or functional silencing (clonal anergy) to ensure peripheral tolerance to self antigens (Goodnow *et al.*, 1988; Nemazee and Burki, 1989; Hartley *et al.*, 1993). All of these findings indicate that cell death plays a pivotal role in the selection of B cell populations during B cell maturation. The developmental program must be tightly controlled since it has to promote the generation of functional IgM$^+$ B cells but, at the same time, the demise of self-reactive B cells.

Little is currently known about the molecular events that regulate the survival of developing B cells. A clear candidate for such a function is the product of the *bcl-2* proto-oncogene. Deregulated expression of Bcl-2 blocks apoptosis, a form of physiological cell death commonly occurring during lymphoid and neuronal development (McDonnell *et al.*, 1989; Sentman *et al.*, 1991; Strasser *et al.*, 1991a,b; Garcia *et al.*, 1992). Importantly, overproduced Bcl-2 prevents the death of B cells cultured *in vitro* (Nuñez *et al.*, 1990; Strasser *et al.*, 1991a) and its expression is absent or diminished in germinal centers, sites associated with extensive apoptotic B cell death (Pezzella *et al.*, 1990; Hockenbery *et al.*, 1991). Together these findings suggest that Bcl-2 may be involved in modulating the survival pathway of developing B cells. However, the expression of Bcl-2 in developing B cells and its precise normal role remains uncertain because previous experiments were performed in growth-factor dependent cell lines transfected with *bcl-2* gene constructs (Nuñez *et al.*, 1990) or in transgenic mice overexpressing Bcl-2 in the B cell lineage (McDonnell *et al.*, 1989; Strasser *et al.*, 1991a). Our results

demonstrate a highly restricted regulation of the Bcl-2 protein during B cell development. Furthermore, we find that susceptibility to cell death induced by the apoptosis-inducing hormone dexamethasone is stage-dependent and correlates with the amount of Bcl-2 expressed by individual B cells, arguing for a physiological role of Bcl-2 in B cell development.

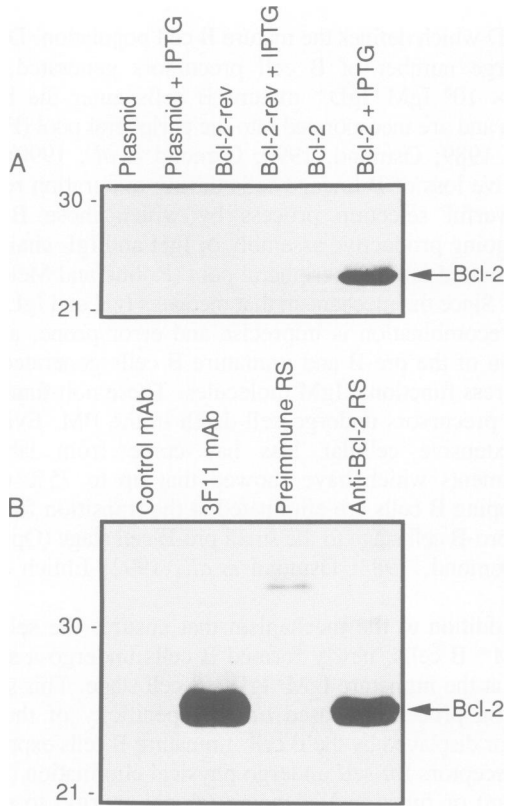

Fig. 1. Recognition of the murine *bcl-2* gene product by the 3F11 mAb. (**A**) Western blot analysis of whole-cell lysates of bacteria transformed with the prokaryotic expression vector PET-3d alone, PET-3d containing a truncated mouse *bcl-2* cDNA lacking the C-terminal 22 amino acids in antisense orientation (Bcl-2-rev), or PET-3d with the same *bcl-2* insert in sense orientation (Bcl-2). Lysates were prepared from uninduced cultures or from bacteria induced with IPTG. (**B**) Immunoprecipitation of the Bcl-2 murine product by the 3F11 mAb. BAL-17 B cells were metabolically labelled with [^{35}S]methionine-cysteine and lysates were immunoprecipitated with control hamster mAb, 3F11 mAb, preimmune rabbit serum or rabbit anti-Bcl-2 serum.

Results

The monoclonal antibody 3F11 recognizes the murine bcl-2 gene product

To assess the expression of Bcl-2 during lymphoid development, a monoclonal antibody (mAb), 3F11, specific for the murine *bcl-2* gene product was generated by immunizing hamsters with purified recombinant Bcl-2 protein expressed in *Escherichia coli* (Veis *et al.*, 1993). The reactivity of the 3F11 mAb was studied by Western blot analysis of whole-cell lysates from *E.coli* transformed with an expression vector containing a truncated mouse *bcl-2* cDNA product lacking the C-terminal 22 amino acids. Figure 1A shows that 3F11 mAb recognized a 23 kDa band corresponding to the truncated *bcl-2* gene product in lysates from bacteria transformed with the *bcl-2* cDNA. Expression of the Bcl-2 protein was strongly enhanced by induction with isopropyl-β-D-thiogalactopyranoside (IPTG). However, the 23 kDa product was not detected in lysates from bacteria transformed with the vector alone or with the expression plasmid containing the same *bcl-2* insert in antisense orientation (Bcl-2-rev). In addition, 3F11 immunoprecipitated the endogenous 25 kDa *bcl-2* gene product from ^{35}S-labelled BAL-17 murine lymphoma B cells (Figure 1B). The same 25 kDa band was observed using a rabbit polyclonal antibody raised against a murine Bcl-2 fusion protein (Cuende *et al.*, 1993), but not with control hamster mAb or preimmune rabbit serum (Figure 1B). Finally, 3F11 mAb fails to recognize murine Bcl-x protein (data not shown), which presents a high homology with Bcl-2 (M.González-García and G.Nũnez, in preparation).

The Bcl-2 protein is differentially expressed in immature and mature B cells

We have previously shown a differential expression of *bcl-2* RNA in immature and mature B-cell lymphoma lines (Cuende *et al.*, 1993), suggesting that the *bcl-2* gene may be developmentally regulated in B lymphocytes. To study Bcl-2 expression in detail, the levels of Bcl-2 protein were examined in BM B-lineage cells of 6–8 week-old B6 mice by three-color flow cytometric analysis. We first examined the expression of Bcl-2 in mature and immature BM B cells. Both B cell populations are characterized by the expression of membrane IgM. However, surface IgD distinguishes immature from mature B cells (Goodnow *et al.*, 1988). Figure 2 shows that the great majority of IgM$^+$IgD$^+$ B

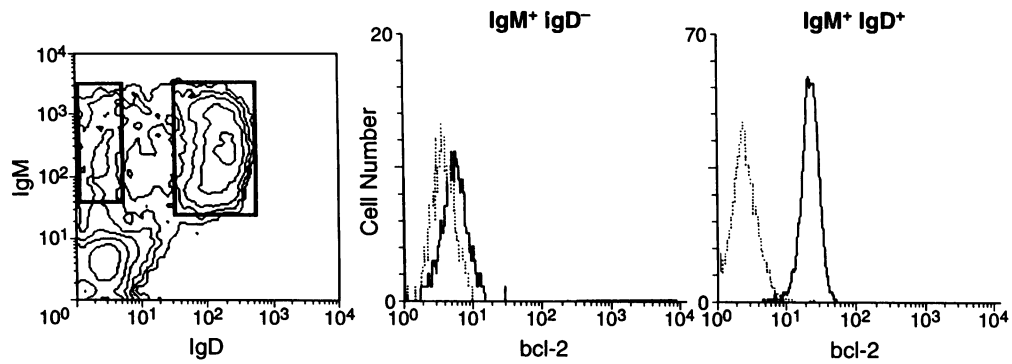

Fig. 2. Expression of the Bcl-2 protein in immature and mature B cells. BM cells from adult B6 mice were stained with anti-IgD, anti-IgM or 3F11 mAb. Left panel shows staining for surface IgM and IgD. The middle and right panels represent the intensity of Bcl-2 staining (solid line) in the IgM$^+$IgD$^-$ (center) and IgM$^+$IgD$^+$ (left) B cell populations. Background staining with control hamster IgG mAb is shown for comparison (dotted line). Results are representative of five independent experiments.

cells (97 ± 3%, n = 5) express high amounts of Bcl-2 protein. By contrast, >90% (95 ± 4%, n = 5) of the IgM^+IgD^- immature B cells displayed undetectable or very low levels of the Bcl-2 protein. In addition, the expression of Bcl-2 was compared between mature B cells from BM and peripheral (splenic and peritoneal) conventional (IgM^+CD5^- cells) and $CD5^+$ (IgM^+CD5^{dull} cells) B lymphocytes. No differences were observed in the levels of Bcl-2 displayed by all these three B cell populations (data not shown).

Bcl-2 is highly expressed in early B cell progenitors and is downregulated in pre-B cells

Several studies have shown that during B cell development, the greatest cell loss occurs at the transition between pro-B to pre-B cells (Opstelten and Osmond, 1983; Ehlich *et al.*, 1993). Therefore, it was important to assess Bcl-2 expression in early B cell progenitors. Both pre-B and pro-B cells are characterized by the absence of surface IgM and by the expression of low levels of the CD45/B220 molecule (Forster *et al.*, 1989; Hardy *et al.*, 1991; Ehlich *et al.*, 1993). When

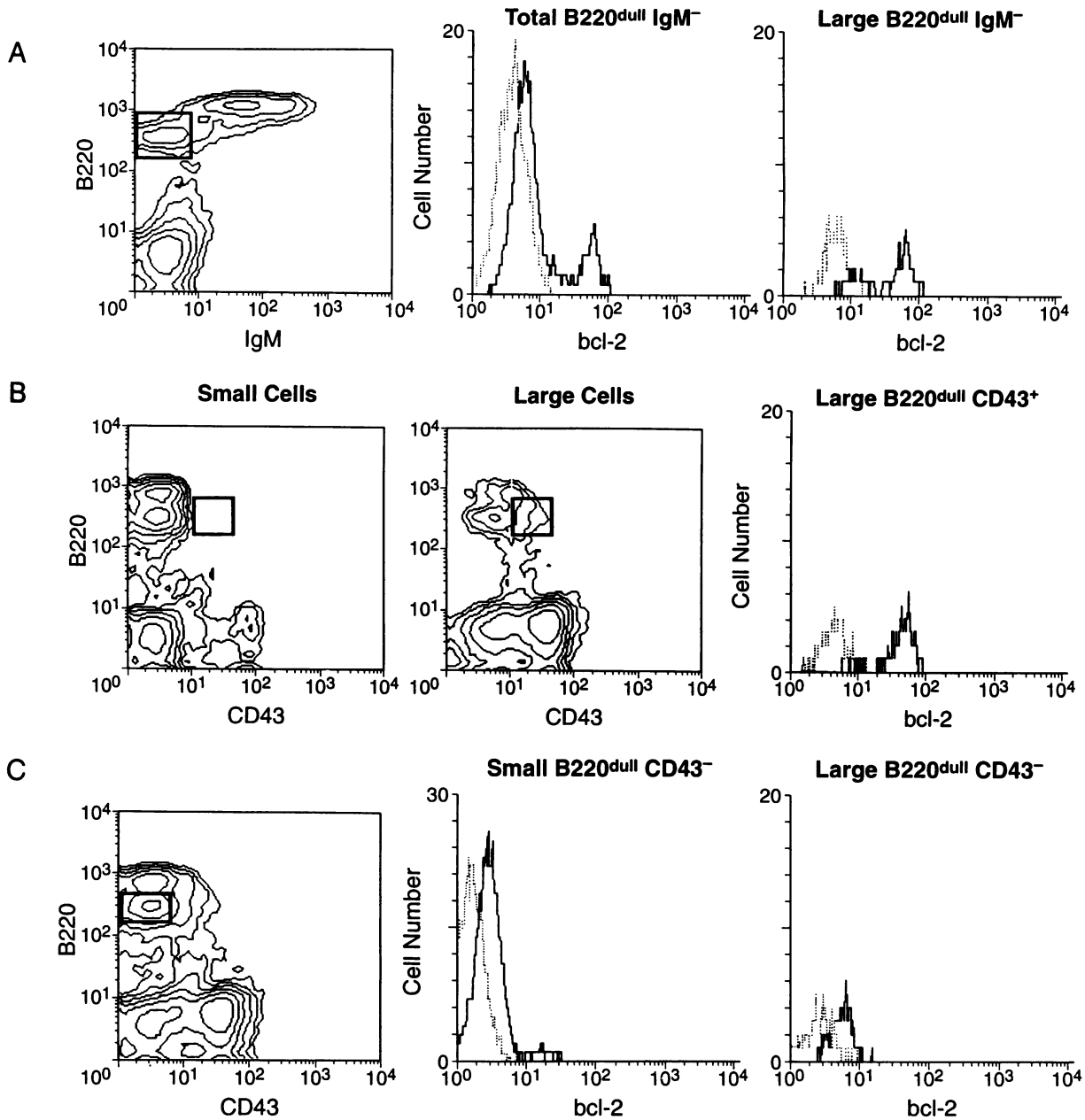

Fig. 3. Expression of Bcl-2 in early B cell precursors. (**A**) Three-color flow cytometric analysis of B220, IgM and Bcl-2 in BM of adult mice. Left panel represent contour-plots of BM cells stained with anti-B220 and anti-IgM mAb. The expression of Bcl-2 (solid line) in the $B220^{dull}IgM^-$ cell population is shown in the center (total $B220^{dull}IgM^-$ cells) and right (large size $B220^{dull}IgM^-$ cells) panels. Dotted lines represent background fluorescence. Results are representative of five independent experiments. (**B**) Expression of Bcl-2 in $B220^{dull}CD43^+$ pro-B cells. Contour-plots of the small (left panel) and large (center panel) BM cells stained with anti-B220 and anti-CD43 mAb. The expression of Bcl-2 in large $B220^{dull}CD43^+$ pro-B cells is shown in the right panel (solid line). Separation between small and large cells was based on their forward-scatter profile. Background fluorescence is shown for comparison (dotted line). Results are representative of five independent experiments. (**C**) Expression of Bcl-2 in $B220^{dull}CD43^-$ B cells. Left panel represents contour-plots of BM cells stained with anti-B220 and anti-CD43 mAb. The expression of Bcl-2 (solid line) in the small and large BM cells is shown in the center and right panels, respectively. Background staining with control hamster IgG mAb is shown for comparison (dotted line). Results are representative of five independent experiments.

Table I. Phenotypic distribution of BM B cell populations in dexamethasone-treated or control mice

B cell population	Treatment[a]	% of B220$^+$ cells[b]	No. of cells ($\times 10^5$)[c]	% Bcl-2$^+$ cells[b]	% Bcl-2$^-$ cells[b]
IgM$^+$IgD$^+$	–	30 ± 10	46.8 ± 19.7	97 ± 3	2 ± 1
(Mature B cells)	Dex	67 ± 4	26.4 ± 10.6 (1.7)[d]	98 ± 1	<1
IgM$^+$IgD$^-$	–	15 ± 3	22.9 ± 6.5	4 ± 1	95 ± 4
(Immature B cells)	Dex	7 ± 1	2.4 ± 0.8 (9.5)	30 ± 5	66 ± 6
B220^{dull}IgM$^-$	–	41 ± 10	62.3 ± 16.3	15 ± 2	83 ± 5
(Pro-B and pre-B cells)	Dex	13 ± 5	4.3 ± 2.2 (15.4)	82 ± 4	16 ± 3
B220^{dull}CD43$^+$	–	9 ± 2	10.1 ± 2.2	85 ± 6	12 ± 2
(Pro-B cells)	Dex	12 ± 4	4.1 ± 0.6 (2.4)	98 ± 1	<1

[a]B6 mice (6–8 weeks old) received an i.p. injection of 2 mg dexamethasone. As controls, untreated or PBS-treated mice were used. Two days after treatment, BM cells were harvested and stained. No differences in the percentage and number of B cells were observed between untreated and PBS-treated control mice.
[b]Results are expressed as mean (three to five mice per group) ± 1 SD of cells in each B cell-lineage population.
[c]Absolute number of cells per two femora and two tibiae. Results are expressed as mean (three to five mice per group) ± 1 SD.
[d]Differential ratio in the number of B-lineage cells between control and dexamethasone-treated mice.

BM cells were stained with mAbs specific for IgM, B220 and Bcl-2, two B220^{dull}IgM$^-$ B cell populations expressing different amounts of Bcl-2 were clearly identified (Figure 3A). One population which represented the great majority of the cells (83 ± 5%, n = 5) exhibited undetectable or very low levels of Bcl-2 protein. By contrast, the second population of B220^{dull}IgM$^-$ B cells which comprised 15% of the cells displayed levels of Bcl-2 that were similar to those observed in mature IgM$^+$IgD$^+$ B lymphocytes (Figure 3A). Since the B220^{dull}IgM$^-$ B cell population is comprised of less mature large pro-B cells and more mature small pre-B cells (Forster *et al.*, 1989; Hardy *et al.*, 1991; Ehlich *et al.*, 1993), the expression of Bcl-2 was examined in both the small and large-sized cell fractions of BM B220^{dull}IgM$^-$ B cells. Significantly, Bcl-2^{bright} B cells were almost exclusively identified in the fraction of large B220^{dull}IgM$^-$ B cells with <3% of the small cells expressing high Bcl-2 levels (Figure 3A). These results suggested that the Bcl-2^{bright} cells observed in the B220^{dull}IgM$^-$ B cell population correspond to pro-B cells. To assess that more directly, BM cells were labelled with anti-CD43, the murine homologue of leukosialin which has been shown to be expressed at low levels in pro-B cells but not in pre-B cells (Hardy *et al.*, 1991; Ehlich *et al.*, 1993). In agreement with previous reports (Hardy *et al.*, 1991; Ehlich *et al.*, 1993), the B220^{dull}CD43$^+$ cells were almost exclusively found in the fraction of large BM cells (Figure 3B). Importantly, the majority of the B220^{dull}CD43$^+$ cells (85 ± 6%, n = 5) displayed high levels of Bcl-2 protein. In contrast, <5% of the B220^{dull}CD43$^-$ (pre-B and immature B cells) were Bcl-2^{bright} cells (Figure 3C). These results demonstrate a biphasic regulation of Bcl-2 in developing B cells. Bcl-2 is highly expressed in the great majority of large CD43$^+$ pro-B cells, low or undetectable in pre-B and immature IgM$^+$IgD$^-$ B cells and upregulated in mature IgM$^+$IgD$^+$ B cells. The number of B cell precursors and the percentage of Bcl-2^{bright} cells in each BM B cell population is summarized in Table I.

Bcl-2 is a long-lived protein

Previous *in vivo* 5-bromo-2′-deoxyuridine labelling experiments indicated that the renewal of early BM B cell precursors is extremely high (Forster *et al.*, 1989; Hartley *et al.*, 1993). In these studies it has been estimated that the half-life of early B cell precursors is ~1 day. The down-modulation of the Bcl-2 protein during B cell development raises the question of how Bcl-2 is regulated in developing B cells. To begin to analyze this, we assessed the turnover of the Bcl-2 protein in B cells. BAL-17 cells that display a mature B cell phenotype were pulse-labelled for 1 h with [^{35}S]methionine-cysteine and chased for different times, and lysates were immunoprecipitated with anti-Bcl-2 polyclonal antibody (Cuende *et al.*, 1993). As shown in Figure 4, the Bcl-2 protein has a relatively long half-life of ~10 h. These results, together with the recent finding that pre-B cells express lower amounts of *bcl-2* mRNA than mature B cells (Haury *et al.*, 1993; Li *et al.*, 1993), suggest that Bcl-2 expression in developing B cells is regulated by transcriptional mechanisms.

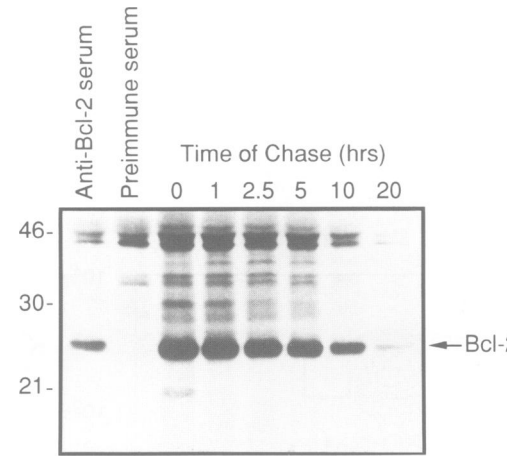

Fig. 4. Determination of Bcl-2 protein half-life. [^{35}S]methionine-cysteine-labelled BAL-17 cells were chased for the times indicated. Cell lysates were then immunoprecipitated with an anti-Bcl-2 polyclonal antibody. Immunoprecipitated proteins were resolved by SDS–PAGE and detected by autoradiography. First two lanes show labelled cell lysates immunoprecipitated with anti-Bcl-2 polyclonal antibody and preimmune serum as controls. The number of counts loaded was approximately four times lower in control lanes than in chased samples.

Selective elimination of Bcl-2^{dull} B cell progenitors following treatment with dexamethasone

The results shown above indicate a high correlation between diminished Bcl-2 expression and those stages of B cell

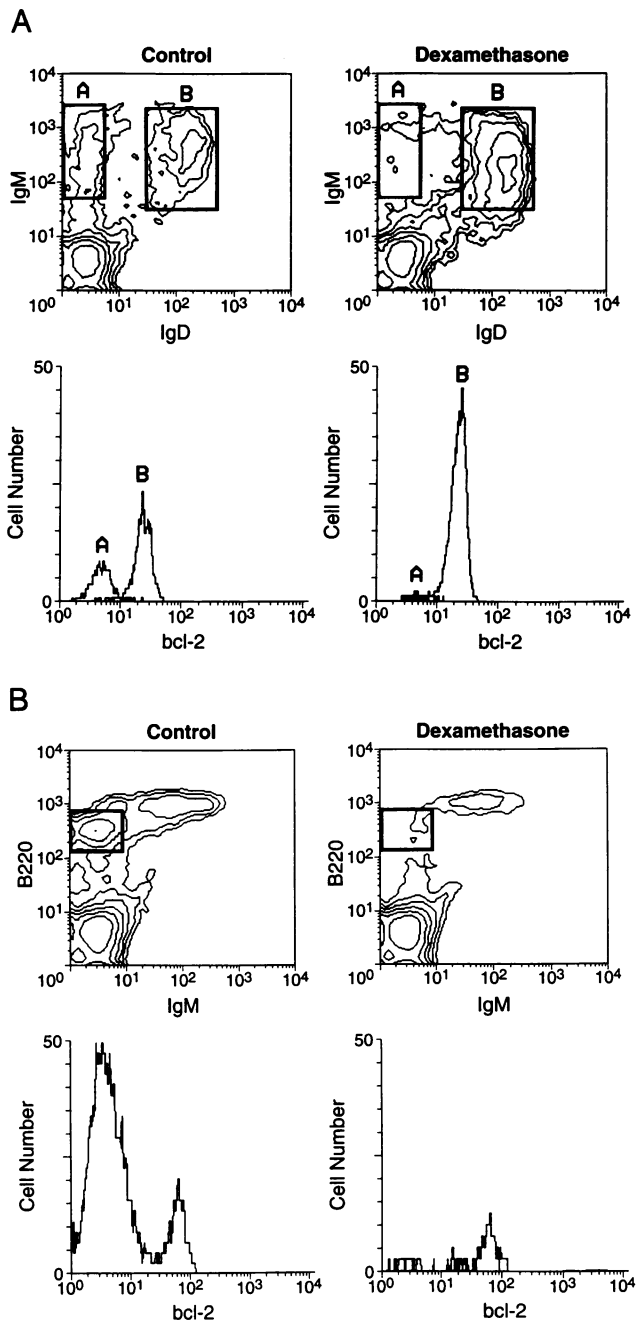

Fig. 5. Dexamethasone induces depletion of BM B cells expressing little or no Bcl-2. (**A**) Flow cytometric analysis of IgM, IgD and Bcl-2 in BM cells from adult mice treated with PBS or dexamethasone. Upper panels represent contour-plots of BM cells stained with anti-IgM and anti-IgD from PBS-treated (left) and dexamethasone-treated (right) mice. The expression of Bcl-2 in the IgM^+IgD^- (peak A) and IgM^+IgD^+ (peak B) gated B cells in PBS-treated (left) and dexamethasone-treated (right) mice is shown in the lower panels. Results are representative of three independent experiments. (**B**) Effect of dexamethasone treatment in $B220^{dull}IgM^-$ BM cells. Contour-plots of $B220^{dull}IgM^-$ BM cells from PBS-treated (left) and dexamethasone-treated (right) mice are shown in upper panels. Lower panels show levels of Bcl-2 in control and dexamethasone-treated mice. The $B220^{dull}IgM^-$ B cell population that persists after treatment with dexamethasone was identified as $CD43^+$ pro-B cells (not shown). Results are representative of three independent experiments.

development associated with physiological cell death (namely, pre-B and immature B cells). However, it was unclear whether the developmental regulation of the Bcl-2 protein was important in modulating apoptosis in B cell populations. To begin to assess that, adult mice were treated with dexamethasone, a compound known to induce apoptosis in lymphoid cells (Sabbele *et al.*, 1987). In a preliminary experiment, we found that the optimal dose to induce cell death in BM B cells was 2 mg of dexamethasone, in agreement with a previous report (Sabbele *et al.*, 1987). Two days following injection of 2 mg of dexamethasone, there was a >9-fold reduction in the number of IgM^+IgD^- immature B cells when compared with mock-treated mice (Table I). In contrast, IgM^+IgD^+ mature B cells were more resistant to dexamethasone (<2-fold loss) (Table I). Significantly, the large majority of the cells that survived treatment with dexamethasone were IgM^+IgD^+ B cells expressing high levels of Bcl-2 (Figure 5A). When the effect of dexamethasone was examined in early B cell progenitors, there was a 15-fold reduction in the number of $B220^{dull}IgM^-$ B cells relative to PBS-treated mice (Table I). The great majority of $B220^{dull}IgM^-$ B cells (82 ± 4%) that survived dexamethasone treatment expressed high levels of Bcl-2 (Figure 5B and Table I), and the CD43 marker (data not shown) indicating that they were pro-B cells. Furthermore, $B220^{bright}IgM^+$ mature B cells were less susceptible than $B220^{dull}IgM^+$ cells (immature B cells) to dexamethasone treatment (Figure 5B), in agreement with results shown in the Figure 5A. These results indicate that susceptibility to cell death induced by the corticosteroid hormone dexamethasone is stage-dependent in developing B cells and is correlated with the expression of Bcl-2.

Expression of a bcl-2 transgene rescues pre-B and immature B cells from cell death

The precedent results suggest that Bcl-2 is involved in controlling the susceptibility to cell death induced by dexamethasone. To assess more directly the function of Bcl-2 in early B cell progenitors, the response to dexamethasone was analyzed in transgenic mice (McDonnell *et al.*, 1989, 1990) in which Bcl-2 has been targeted to the B-cell lineage. In contrast to normal mice, practically all B cell populations including pre-B and immature B cells from *bcl-2*-Ig transgenic mice express high levels of Bcl-2 (Figure 6). As described above, treatment of normal mice with dexamethasone selectively eliminates pre-B and immature B cell populations as compared with pro-B and mature B cells (Figure 7). However, in *bcl-2*-Ig transgenic mice, overexpression of Bcl-2 protected pre-B and immature B cells from dexamethasone-induced cell death (Figure 7 and Table II). These results indicate that Bcl-2 can inhibit the apoptotic pathway at early stages of B cell development.

Discussion

During B cell development, a highly efficient mechanism is operative to ensure the survival of progenitor B cells capable of differentiating into mature IgM^+IgD^+ B cells. Given the random nature of the immunoglobulin gene recombination process, it has been suggested that the majority of the rearrangements in B cell progenitors would be non-productive (Korsmeyer *et al.*, 1981; Alt and Baltimore, 1982). On the other hand, practically all mature B cells display IgM receptors on their surface, arguing that pre-B cells that fail to express functional immunoglobulin rearrangements are selectively eliminated. How is the selection of functional mature B cells achieved? The results

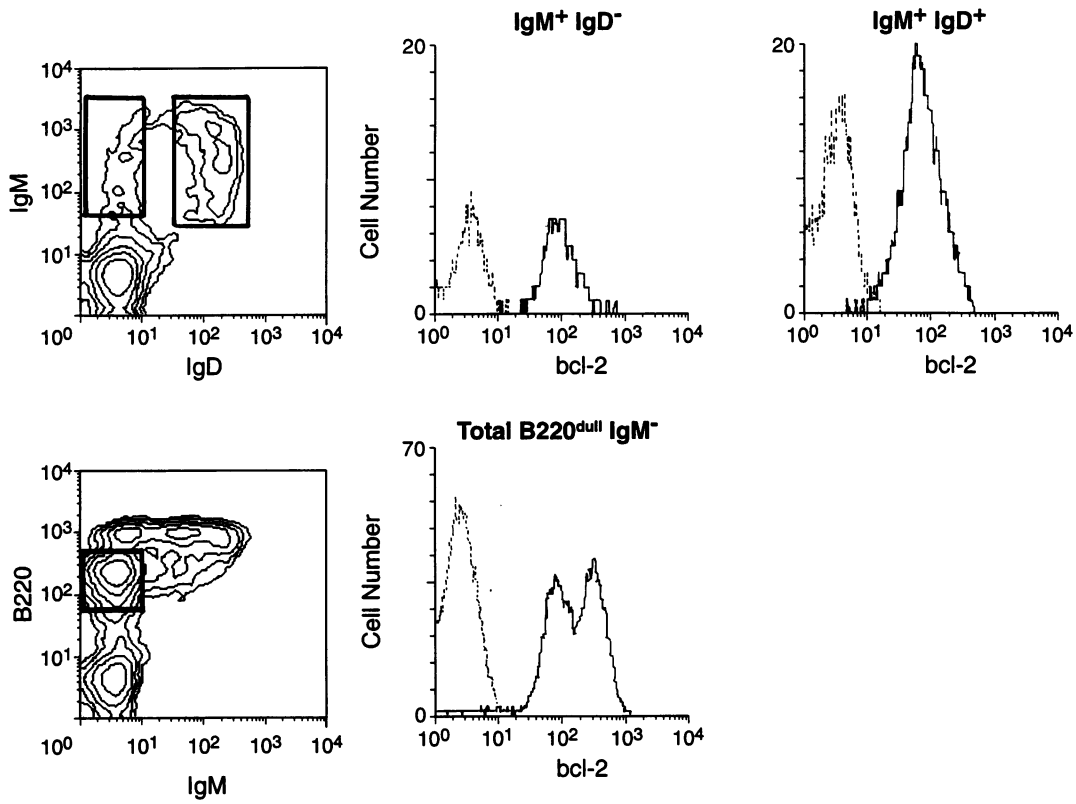

Fig. 6. Expression of a *bcl-2* transgene in BM B cell progenitors. In the upper panels, three-color flow cytometry analysis of BM cells from *bcl-2*-Ig transgenic mice stained with anti-IgM, anti-IgD and 6C8 mAb. Left panel shows staining for surface IgM and IgD. The center and right panels represent the intensity of anti-human Bcl-2 staining (solid line) in the IgM^+IgD^- (center) and IgM^+IgD^+ (right) B cell populations. Background staining with control hamster IgG mAb is shown for comparison (dotted line). In the lower panels, the left panel represents contour-plots of BM cells stained with anti-B220 and anti-IgM mAb. The expression of Bcl-2 (solid line) in the $B220^{dull}IgM^-$ cell population (pro-B and pre-B cells) is shown in the right panel. Note that two populations of $B220^{dull}IgM^-$ cells express the *bcl-2* transgene product at different levels. Further analysis revealed that cells staining more intensely for Bcl-2 were $CD43^+$ pro-B cells (data not shown).

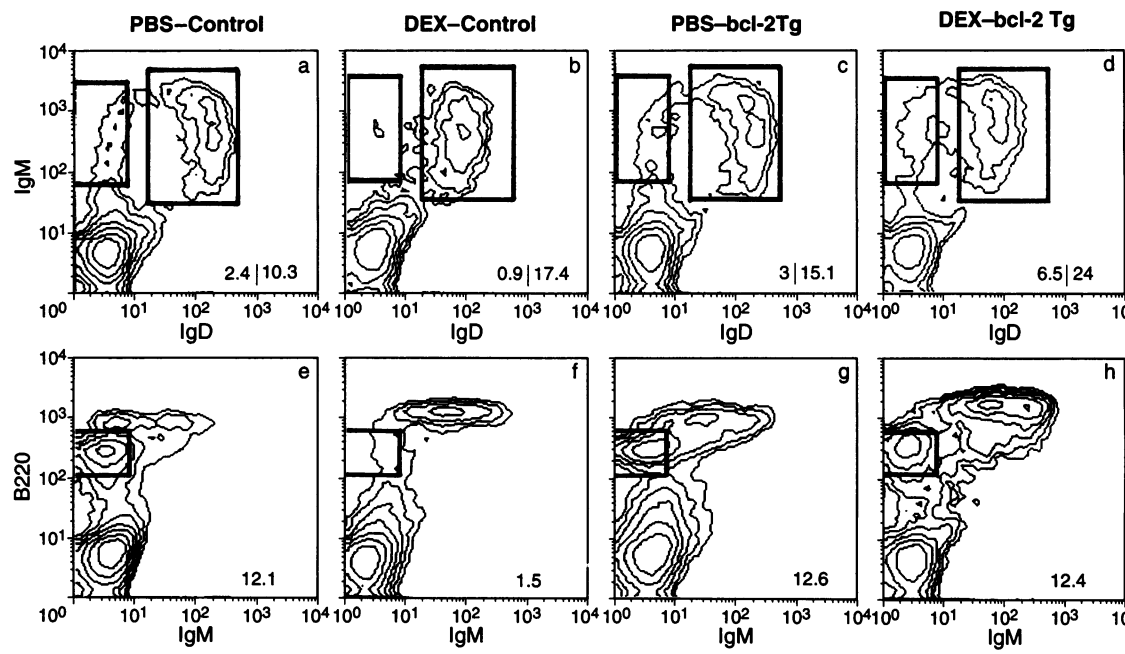

Fig. 7. Expression of a *bcl-2*-Ig transgene rescues B cell progenitors from dexamethasone-induced cell death. BM cells from PBS-treated normal mice (**a** and **e**), dexamethasone-treated normal mice (**b** and **f**), PBS-treated *bcl-2*-Ig transgenic mice (**c** and **g**) and dexamethasone-treated *bcl-2*-Ig transgenic mice (**d** and **h**) were stained with anti-IgM and anti-IgD mAb (a–d) or anti-IgM and anti-B220 mAb (e–h). In upper panels, IgM^+IgD^+ (mature B cells) and IgM^+IgD^- (immature B cells) are boxed. In lower panels, $B220^{dull}IgM^-$ (pro-B and pre-B cells) are boxed. Percentage of cells in each region is shown.

Table II. Phenotypic distribution of BM B cell populations in PBS-treated and dexamethasone-treated *bcl-2*-Ig transgenic mice

B cell population	Treatment[a]	% of B220$^+$ cells[b]	No. of cells ($\times 10^5$)[c]
IgM$^+$IgD$^+$	PBS	55 ± 9	122.5 ± 27.8
(Mature B cells)	Dex.	57 ± 2	119.3 ± 19.4
IgM$^+$IgD$^-$	PBS	11 ± 2	23.4 ± 3.3
(Immature B cells)	Dex.	10 ± 1	20.7 ± 2.4
B220^{dull}IgM$^-$	PBS	21 ± 5	45.7 ± 7.8
(Pro-B and pre-B cells)	Dex.	17 ± 1	38.3 ± 2.1
B220^{dull}CD43$^+$	PBS	11 ± 2	24.0 ± 0.9
(Pro-B cells)	Dex.	10 ± 1	21.1 ± 1.7

[a]B6 and *bcl-2*-Ig transgenic mice (6–8 weeks old) received an i.p. injection of 2 mg of dexamethasone or PBS. Two days after treatment, BM cells were harvested and stained. The results of dexamethasone- or PBS-treated B6 mice are summarized in Table I.
[b]Results are expressed as mean (three mice/group) ± 1 SD of cells in each B cell-lineage population.
[c]Absolute number of cells per two femora and two tibiae. Results are expressed as mean (three mice/group) ± 1 SD.

of the present study suggest that the *bcl-2* proto-oncogone can be involved in the selection of developing B cells. Bcl-2 is highly expressed in the great majority of CD43$^+$ B cell precursors which have been recently showed to undergo rearrangements of IgH and IgL chain genes (Ehlich *et al.*, 1993). The survival of pro-B cells would be critical for the generation of a large pool of pre-B cells expressing a wide array of pre-B receptor complexes formed by the membrane-bound μ chain and the products of the $\lambda 5$ and $V_{pre\text{-}B}$ genes (Karasuyama *et al.*, 1990; Tsubata and Reth, 1990). A role for Bcl-2 in the maintenance of other early progenitor cells in hematopoietic and epithelial tissues has been previously proposed (Hockenbery *et al.*, 1991).

Downregulation of the Bcl-2 protein at the pre-B cell stage coincides with a developmental window characterized by extensive cell death during B cell maturation. Evidence for cell loss at the pre-B cell stage has been provided by several studies. Thus, it has been estimated that as many as 75% of developing B cells are lost at the transition from pro-B to pre-B cell stage and during the pre-B cell stage (Opstelten and Osmond, 1983; Forster *et al.*, 1989; Ehlich *et al.*, 1993). Cell death at the pre-B cell stage probably reflects the elimination of those cells that fail to express a pre-B cell receptor complex. This is supported by a series of elegant experiments using mice in which the μ chain or $\lambda 5$ genes have been disrupted by gene targeting (Kitamura *et al.*, 1991, 1992). These studies have shown that expression of a pre-B cell receptor complex is essential for the maturation of pro-B cells into mature B cells. It has been suggested that expression of a functional IgH chain gene drives B cell survival and differentiation by modulating their growth signal requirements (Era *et al.*, 1991). Taken together, these observations indicate that the presence of a pre-B receptor complex promotes the survival and differentiation of pre-B cells. An alternative interpretation is that the failure to express a pre-B cell receptor complex generates a cell death signal. In the latter model, failure to receive a negative signal at the pro-B/pre-B cell stage would lead to differentiation. Whatever the mechanism, downregulation of Bcl-2 in pre-B cells would facilitate the death of those cells which carry no functionally rearranged immunoglobulin genes. The highly dynamic regulation of Bcl-2 raises the issue of the signal(s) that modulate the levels of Bcl-2 during B cell development. Downregulation of Bcl-2 at the pre-B cell stage may be linked to immunoglobulin gene recombination events or be modulated by cell–cell or growth factor interactions. Our results that Bcl-2 is a relatively long-lived protein, together with the finding of a similar regulation of *bcl-2* at the RNA level in purified BM B cell preparations (Haury *et al.*, 1993; Li *et al.*, 1993), suggest that transcriptional mechanisms are involved in the regulation of Bcl-2 expression during B cell development. This is additionally supported by the recent discovery of a negative regulatory element in the *bcl-2* gene (Young and Korsmeyer, 1993).

At a late stage of development, immature B cells bearing self-reactive IgM molecules are physically eliminated or inactivated by the encounter with self-antigens (Goodnow *et al.*, 1988; Nemazee and Burki, 1989; Hartley *et al.*, 1993). Thus, downregulation of Bcl-2 at the immature B cell stage may enable the deletion of autoreactive B cells. The developmental regulation of Bcl-2 may provide the basis for the differential susceptibility of immature and mature B cells to anti-IgM signalling and tolerance induction. Thus, several studies have shown that signalling via IgM receptors generates a negative signal leading to apoptotic cell death in immature B cells but induces proliferation in mature B cells (Nossal, 1983; Hasbold and Klaus, 1990). One model of clonal selection would propose that deletion of self-reactive immature B cells triggered by the engagement of IgM receptors would be facilitated by diminished expression of Bcl-2. On the other hand, immature B cells that do not encounter self antigens would escape death, upregulate IgD and Bcl-2 expression and exit the BM as mature IgM$^+$IgD$^+$ B cells. We cannot exclude the possibility that Bcl-2 is upregulated during the migration of B cells from the BM to the periphery or at peripheral tissues. This latter interpretation would be also compatible with the presence of mature B cells expressing high levels of Bcl-2 in the BM as a large fraction of those cells recirculate from the periphery to the BM (Forster *et al.*, 1989).

In the human, the expression of Bcl-2 protein is absent or diminished in mature B cells of the germinal centers (Pezzella *et al.*, 1990; Hockenbery *et al.*, 1991), a site associated with a high rate of apoptosis in B cells undergoing somatic hypermutation of immunoglobulin genes. In contrast, upregulation of Bcl-2 in the apical light zone of the germinal centers may be involved in the rescue of B cell mutants that express high-affinity receptors for antigen (Berek *et al.*, 1991; Jacob *et al.*, 1991). These findings indicate that downregulation of Bcl-2 is a common feature of different stages of B cell development characterized by extensive cell death and clonal selection. A restricted expression of the Bcl-2 protein has also been observed in human T cells during their development in the thymus,

indicating that the restricted regulation of Bcl-2 is common to both B and T lymphoid lineages. Thus, Bcl-2 is downregulated in immature $CD4^+CD8^+$ thymocytes, a stage with a high rate of cell death, and upregulated in mature single positive thymocytes and peripheral T cells (Pezzella *et al.*, 1990; Hockenbery *et al.*, 1991; Gratiot-Deans *et al.*, 1993; Veis *et al.*, 1993).

Our results have demonstrated that the susceptibility to cell death induced by dexamethasone, a corticosteroid hormone known to trigger apoptosis, is developmentally regulated in B lymphocytes. Thus, pre-B and immature B cells are highly sensitive to treatment with dexamethasone whereas pro-B and mature B cells are much more resistant. Importantly, there was a high degree of correlation between susceptibility to cell death and the amount of Bcl-2 expressed by individual B cells, suggesting that Bcl-2 expression modulates the response to corticosteroid hormones. The finding that targeting of a *bcl-2* transgene rescued pre-B cells and immature B cells from dexamethasone-induced cell death indicates that Bcl-2 can regulate that response in early progenitors and early B cells. A differential susceptibility to cell death mediated by corticosteroid hormones has been previously demonstrated in thymocytes (Blomgren and Andersson, 1970; Cohen and Duke, 1984). Consistent with our findings in B cells, immature $CD4^+CD8^+$ thymocytes which express low levels of Bcl-2 are highly sensitive to dexamethasone-induced cell death. In contrast, mature single positive thymocytes which display high levels of Bcl-2 are more resistant (Blomgren and Andersson, 1970; Cohen and Duke, 1984). Previous experiments have shown that apoptosis induced by corticosteroid hormones in susceptible lymphoid cells can be inhibited by the overexpression of Bcl-2 from transfected gene constructs or transgenes (Sentman *et al.*, 1991; Strasser *et al.*, 1991b; Alnemri *et al.*, 1992; Miyashita and Reed, 1992). Our observations strongly suggest that endogenous levels of Bcl-2 are important in controlling susceptibility to apoptosis. In addition to *bcl-2*, two recently described genes, *bcl-x* and *bax*, appear to control cell death and Bcl-2 function (Boise *et al.*, 1993; Oltvai *et al.*, 1993). Thus, regulation of apoptosis in developing B cells may be complex and modulated by the interaction of several gene products. In addition to cell death associated with clonal selection events, Bcl-2 may be involved in the protection of $CD43^+$ B cell progenitors and mature B cells from a variety of apoptosis-inducing stimuli including glucocorticoid hormones which are highly secreted under physiological conditions such as stress (Kagan *et al.*, 1989). Conversely, diminished expression of Bcl-2 at specific stages of B cell development may facilitate their apoptotic death triggered by DNA damage or abnormal cell cycle events (Collins *et al.* 1992).

Materials and methods

Animals and treatment

C57BL/6 (B6) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The *bcl-2*-Ig transgenic mice have been described previously (McDonnell *et al.*, 1989, 1990). Animals were between 6 and 8 weeks old when analyzed. To study the effects of dexamethasone on BM B cell populations, B6, *bcl-2*-Ig transgenic or control littermates received a single i.p. injection of 2 mg of dexamethasone sodium phosphate (American Regent Laboratories, Shirley, NY) or phosphate-buffered saline (PBS) as a control. 48 h later BM cells were harvested from both femora and tibiae and stained as described below.

Antibodies

A phycoerythrin (PE)-conjugated rat anti-mouse B220 mAb (clone RA3-6B2), a PE- and fluorescein isothiocyanate (FITC)-labelled mouse anti-mouse IgM^b allotype-specific mAb (AF6-78.25.4), a FITC-conjugated rat anti-mouse leukosialin (CD43) mAb (clone S7), a PE-conjugated rat anti-mouse CD5 mAb (clone 53-7.3) and a hamster anti-trinitrophenyl IgG mAb were purchased from Pharmingen (San Diego, CA). A FITC-conjugated rat anti-IgD mAb was a gift of Dr F.Finkelman (Uniformed Services, University of Health Sciences, Bethesda, MD). A hamster anti-mouse Bcl-2 antibody, clone 3F11, was prepared by immunizing hamsters with purified murine Bcl-2 protein isolated from *E.coli* transformed with the expression vector pMOM 5515 (Olins *et al.*, 1988) in which the mouse *bcl-2* cDNA was cloned (Veis *et al.*, 1993). The 6C8 mAb (hamster anti-human Bcl-2) has been described previously (Hockenbery *et al.*, 1991). A polyclonal rabbit anti-mouse Bcl-2 antibody was produced as described by Cuende *et al.* (1993). Biotinylated $F(ab')_2$ goat anti-hamster IgG was purchased from Jackson ImmunoResearch and streptavidin-RED670™ from Gibco BRL (Gaithersberg, MD).

Flow cytometric analysis

Single cell suspensions from spleen and BM were prepared as previously described (Forster *et al.*, 1989). Peritoneal cells were washed out of the peritoneal cavity with 10 ml of ice-cold PBS containing 5% fetal calf serum (FCS). For three-color staining, 10^6 cells were incubated for 30 min on ice with different combinations of FITC- and PE-labelled mAbs directed against surface B cell antigens. After washing twice with 1% bovine serum albumin (BSA) in PBS, cells were fixed with 1% paraformaldehyde for 10 min at room temperature and stained for Bcl-2. After fixation cells were incubated with 3F11, 6C8 or control hamster mAb in 0.3% saponin in PBS for 30 min at 4°C, washed twice with 0.03% saponin/PBS and incubated with $F(ab')_2$ biotinylated goat anti-hamster IgG for 30 min at 4°C. Finally, cells were washed with 0.03% saponin in PBS, and incubated with streptavidin-RED670™. Cells were analyzed in a FACScan flow cytometer using Lysis II software (Beckton Dickinson, Mountain View, CA). Dead cells and debris were gated out based on forward and sideways light scatter. The results are presented as the log fluorescence intensity of 5×10^4 viable cells.

Generation of bcl-2 gene constructs

A complementary DNA containing a truncated murine *bcl-2* coding sequence was inserted into the *Nco*I site of the PET-3d prokaryotic expression vector (Rosenberg *et al.*, 1987). The truncated *bcl-2* gene was obtained by polymerase chain reaction, using the complete *bcl-2* cDNA as a template and modified primers. The 5′ end primer contained an added *Nco*I site for direct cloning (5′-GAACCATGGCGCAAGCCGGGAGA-3′). The 3′ end primer introduced a stop codon at position 218 followed by an *Nco*I site (5′-CAGCCATGGTCACAGAGACAGCCAGGAGAA-3′). The sequence of the *bcl-2* gene construct was confirmed by dideoxy-chain termination sequencing. Expression of the Bcl-2 protein in *E.coli* was performed as described by Rosenberg *et al.* (1987).

Western blot, immunoprecipitation and pulse–chase analysis

The BAL-17 murine B-cell lymphoma cell line was maintained in RPMI-1640 supplemented with 10% FCS, antibiotics and 50 μM 2-mercaptoethanol. For immunoprecipitation experiments, cells were labelled with [^{35}S]methionine-cysteine (ICN Biomedicals) for 4 h at 37°C. For each sample, lysates from 3×10^6 cells were produced by incubation in lysis buffer (PBS containing 1% Triton X-100, 0.1% SDS and 0.5% deoxycholic acid) for 1 h at 4°C. Immunoprecipitation was carried out by incubating lysates with mAb 3F11, an irrelevant hamster mAb, a polyclonal rabbit anti-Bcl-2 antibody or preimmune rabbit serum from the same rabbit for 15 h at 4°C. After incubation with protein A–Sepharose (Pharmacia) for 1 h at 4°C, immune complexes were collected by centrifugation at 10 000 *g* for 15 s at 4°C. Immune complexes were washed three times with lysis buffer and suspended in sample buffer (0.2% SDS, 10% glycerol, 10 mM Tris pH 6.8 and 0.001% bromophenol). After heating at 85°C for 10 min, samples were loaded onto a polyacrylamide gel. Western blot analysis of whole bacterial cells using the 3F11 mAb was performed essentially as described previously (Veis *et al.*, 1993). Detection of Western blot reactions was performed by incubation with a goat antibody against hamster horseradish peroxidase (McDonnell *et al.*, 1990), and developed with diazobenzidine containing 0.03% nickel chloride (McDonnell *et al.*, 1990). For pulse–chase experiments BAL-17 cells were metabolically labelled with [^{35}S]methionine-cysteine for 1 h at 37°C. After extensive washing, labelled cells were incubated in complete culture medium for the duration of the chase. At each time point, cells containing equal numbers of counts were lysed and Bcl-2 was immunoprecipitated with a polyclonal rabbit anti-Bcl-2

antibody as described above. The half-life of Bcl-2 was determined by laser densitometry scanning. The turnover of total proteins was determined by TCA precipitation of ^{35}S-labelled cell extracts.

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