The bcl-2 Gene Product Inhibits Clonal Deletion of Self-reactive B Lymphocytes in the Periphery but Not in the Bone Marrow

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Summary

To test whether the product of the bcl-2 proto-oncogene blocks clonal deletion of self-reactive B cells, we have generated transgenic mice carrying the bcl-2 gene and the immunoglobulin genes for the anti-erythrocyte 4C8 antibody. In these transgenic mice, clonal deletion of self-reactive immature B cells in the bone marrow was not inhibited in spite of expression of the bcl-2 gene. In contrast, self-antigen-induced clonal deletion of mature self-reactive Ly-1 B (B1) cells in the peritoneal cavity was inhibited in the transgenic mice. These results indicate that the mechanism for clonal deletion of immature self-reactive B cells in the bone marrow differs from that of mature self-reactive B cells in the periphery.

Ig V gene diversity is amplified by somatic DNA rearrangement in B cell precursors of the bone marrow and fetal liver, and by somatic mutations in mature B cells of the peripheral lymphoid organs. Such amplification of diversity allows the immune system to react with almost all possible foreign antigens. The Ig repertoire amplified by genetic mechanisms, includes Ig reactive to self as well as foreign antigens and thus, without appropriate selection, self-reactive B cells inevitably emerge.

Studies on autoantibody transgenic mice (1–5) have clearly demonstrated that self-reactive B cells are surveyed by multiple mechanisms of immunological tolerance (6–8). Soluble self-antigens render self-reactive B cells to an inactive state, in which B cells fail to respond to the stimulation by antigens and T helper cells (clonal anergy). Membrane-bound self-antigens eliminate self-reactive B cells presumably by strong cross-linking of surface Ig (slg)1 receptors (clonal deletion). In transgenic mice which produce autoantibodies reactive to self-antigens expressed in the bone marrow, both bone marrow and peripheral B cells are tolerized by either anergy or deletion, suggesting that the bone marrow immature B cells may be the major target of the B cell tolerance (1, 3, 5). However, the transfer experiment of mature self-reactive B cells into mice producing self-antigens revealed that mature B cells can be also anergized (1). Since self-antigens in the liver eliminated self-reactive B cells in the periphery but not in the bone marrow (9), clonal deletion also appears to take place at some stage after B cells migrate from the bone marrow to the periphery.

We have analyzed a transgenic mouse line expressing both H and L chains of the 4C8 antierthrocyte autoantibody in almost all the B cells (5). As is the case for other autoantibody transgenic mice (1, 3), the number of B cells in the bone marrow, spleen, and lymph nodes in these transgenic mice are markedly reduced most likely because of clonal deletion at the immature B cell stage in the bone marrow by interaction with RBCs and/or their precursors. However, mature Ly-1 B cells reacting to RBCs are found in the peritoneal cavity presumably because the peritoneal cavity is inaccessible from self-antigens (RBCs). These mature Ly-1 B cells undergo apoptotic cell death upon intraperitoneal injection of RBCs (10). Thus, both immature bone marrow B cells and mature Ly-1 B cells are eliminated by interaction with the self-antigens in these mice.

Accumulating evidence suggests that bone marrow immature B cells and peripheral mature B cells are functionally distinct. Immature B cells do not respond to cross-linking of the slg in vitro, whereas the same stimulation induces DNA synthesis in mature B cells (11). Further evidence comes from the observation that slg-associated molecules and signal transduction pathways via slg receptor complexes are different in immature and mature B cells (12). In spite of these differences, no data refer to distinct tolerance mechanisms between B cells in the bone marrow and the periphery.

The bcl-2 proto-oncogene was found as a result of its trans-
location to the Ig H chain locus in most human follicular center B cell lymphomas (13). The bcl-2 gene product has been shown to delay the onset of apoptotic cell death in certain hematopoietic cell lines deprived of growth factors (14–17). Besides the inhibition of apoptosis in these cell lines, the bcl-2 gene product also inhibits apoptosis of normal lymphocytes. Indeed, Strasser et al. (18) and Sentman et al. (19) found that anti-CD3 antibody-induced apoptosis of immature thymocytes was inhibited in bcl-2 transgenic mice. These transgenic mice, however, showed little, if any, abrogation of clonal deletion of T cells reactive to self-superantigens, suggesting that self-tolerance is maintained by multiple mechanisms, some of which are not perturbed by bcl-2.

To assess whether bcl-2 inhibits elimination of self-reactive B cells in both the periphery and the bone marrow, we analyzed antigen-induced elimination of peripheral Ly-1 B cells and clonal deletion of immature B cells in the bone marrow in transgenic mice carrying the genes for bcl-2 and the antilythrocyte antibody. The data shown here demonstrate that the bcl-2 gene product inhibits clonal deletion of peritoneal Ly-1 B cells but not that of bone marrow immature B cells, suggesting that distinct mechanisms are involved in self-tolerance of B lymphocytes in central and peripheral lymphatic organs.

Materials and Methods

Transgenic Mice. The transgenic mouse lines carrying either Ig μ or L chain gene for antilythrocyte antibody (4C8 mAb) were established as described by Okamoto et al. (5) and maintained in our animal colony. The Eμ-bcl-2 transgenic line was kindly donated by Drs. A. Strasser (18). The transgenic line carrying μ chain gene for anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibody was kindly donated by Drs. T. Era and S.-I. Nishikawa (20).

Flow Cytometry and Cell Sorting. Cells from the spleen, the bone marrow, and the peritoneal cavity of mice were applied to Ficoll-Hypaque gradient centrifugation, and mononuclear cells enriched at the interface layer were used for flow cytometric analysis. Single cell suspensions were stained with appropriate antibodies, and analyzed by FACScan® (Becton Dickinson & Co., Mountain View, CA) after exclusion of dead cells by propidium iodide gating. Two-color staining of the cells with anti-B220 and anti-IgM antibodies was done as described by Okamoto et al. (5). The amounts of autoantibodies on erythrocytes were measured with flow cytometry using FITC goat anti-mouse IgM antibody (Cappd Laboratories, Malvern, PA) (5). Mean fluorescence intensities (MFIs) were obtained with the equation: MFI = 10^Iz/μs2 (X = mean fluorescence).

Polymerase Chain Reaction. Total cytoplasmic RNA was extracted from 10^4 splenocytes or bone marrow cells of mice using the RNase inhibitor vanadyl ribonucleotide complex (21). Random primed first strand CDNA synthesis was carried out using abelson murine leukemia virus (AMV) reverse transcriptase (Seikagaku Kogyo, Tokyo, Japan). The cDNA was amplified with human bcl-2 primers; 5' primer, ACACGCCCCATACGCCGCAAT and 3' primer, CAAGCTCCCACCAGC~CCAAA for 30 cycles on a DNA thermal cycler (Perkin Elmer Corp., Nowalk, CT). The expected size of the amplified band is 522 bp.

ELISA. The numbers of antibody-producing cells in 10^6 cells of the spleen, the bone marrow, the lymph nodes, and the peritoneal cavity from mice at 8 wk of age were analyzed with enzyme-linked immunospot assay using anti-idiotype antibody (S54) as described by Murakami et al. (10).

Induction and Assay of Peritoneal Cell Death. 8-wk-old mice were injected intraperitoneally with 10^9 RBCs in 500 μl of PBS. 12 h after injection, peritoneal cells from each mouse were subjected to flow cytometry and DNA fragmentation assay. DNA was isolated from peritoneal cells as described by Ishida et al. (22). The sample was electrophoresed in a 2% agarose gel with TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). DNA was subsequently visualized by ethidium bromide staining.

Results

Autoimmune Symptoms in Transgenic Mice Carrying bcl-2 and Anti-erythrocyte Autoantibody Genes. To examine the effect of the bcl-2 gene on B cell tolerance, we analyzed transgenic mice expressing both the 4C8 antilythrocyte autoantibody and the bcl-2 gene in B lineage cells (bcl-2 × H × L mice). We assessed the incidence of anemia and the amounts of autoantibodies produced by measuring hematocrit (Ht) and quantitating autoantibodies bound to circulating erythrocytes of 8-wk-old bcl-2 × H × L mice. As a control, we examined transgenic mice carrying only the H and L chain genes for the antilythrocyte antibody (H × L mice) at the same age under the same breeding conditions. Both bcl-2 × H × L and H × L mice showed wide variations of the Ht values and the amounts of autoantibodies on RBCs (Fig. 1, a and b). The Ht value in each transgenic mouse was inversely proportional to the amount of the autoantibody bound to erythrocytes, indicating that the autoantibodies are responsible for hemolytic anemia as previously shown for H × L mice (5).

To identify B cells responsible for the autoimmune disease manifested in two thirds of bcl-2 × H × L mice, we compared the number of B cells in various lymphoid organs in severely anemic (Ht <35%) and nonanemic (Ht >35%), indicating the enhanced occurrence of the autoimmune disease by introduction of the bcl-2 transgene. This finding is also supported by the increased amounts of the autoantibody bound to erythrocytes in bcl-2 × H × L mice as compared with H × L mice. Nevertheless, two thirds of H × L mice and one third of bcl-2 × H × L mice showed neither anemia (Ht <40%) nor autoantibody production (MFI >50), indicating that self-reactive B cells are tolerated at least in some individuals of bcl-2 × H × L mice as well as H × L mice.

To identify B cells responsible for the autoimmune disease manifested in severely anemic (Ht <35%) and nonanemic (Ht >40%) bcl-2 × H × L mice, we compared the number of B cells in various lymphoid organs in severely anemic (Ht <35%) and nonanemic (Ht >40%) bcl-2 × H × L mice. The numbers of B cells in the bone marrow and spleen of bcl-2 × H × L mice were decreased to the same level regardless of their Ht values as shown in H × L mice (10). We next examined the number of autoantibody-producing cells in the spleen, bone marrow, lymph nodes, and peritoneal cavity in bcl-2 × H × L mice. The number of antibody-producing cells in the peritoneal cavity of anemic bcl-2 × H × L mice was markedly increased.
Figure 1. Hemolytic anemia in H × L and bcl-2 × H × L mice. (a) Ht values of H × L and bcl-2 × H × L mice. (O, ●) 18 individuals of H × L mice and 12 individuals of bcl-2 × H × L mice, respectively. Ht value of each individual is indicated. (b) MFIs of H × L and bcl-2 × H × L mice. The amounts of the autoantibody (IgM) bound to circulating erythrocytes were quantitated by staining with anti-lgM antibody as described in Materials and Methods. The MFI of each H × L (O) and bcl-2 × H × L (●) mouse is indicated. All the mice used were maintained under the same conditions.

Table 1. Localization of anti-RBC Antibody-producing Cells in Anemic and Nonanemic bcl-2 × H × L Mice

| Symptom               | Spleen | Bone marrow | Inguinal lymph node | Peritoneum |
|-----------------------|--------|-------------|---------------------|------------|
| Severe anemia         | <10    | <10         | <10                 | 221 ± 25\(^\dagger\) |
| Nonanemia             | <10    | <10         | <10                 | 24.7 ± 5.5\(^\dagger\) |

\(^*\) Numbers of antierythrocyte antibody-producing cells measured by enzyme-linked immunospot assay.
\(^\dagger\) bcl-2 × H × L mice whose Ht values were <35%.
\(^\ddagger\) bcl-2 × H × L mice whose Ht values were >40%.
\(^\dagger\) Mean ± SD of three individuals.

Table 2. Percentage of B Lymphocytes in Various Organs of Transgenic Mice Carrying Either Ig Chain or bcl-2 Gene

| Nontransgenic littermate | L chain | H chain | H × L | bcl-2 | bcl-2 × L | bcl-2 × H | bcl-2 × H × L |
|--------------------------|---------|---------|-------|-------|----------|----------|--------------|
| Spleen                   | 51.2 ± 5.5 | 30.2 ± 3.2 | 36.0 ± 9.2 | 3.4 ± 1.9 | 83.0      | 40.9      | 72.6         | 3.4 ± 1.7   |
| Bone marrow              | 14.4 ± 2.8 | 6.6 ± 1.9  | 10.1 ± 2.1 | 3.1 ± 2.1 | 75.4      | 35.9      | 63.3         | 6.2 ± 2.1   |
| Peritoneal cavity        | 42.5 ± 5.3 | 40.9      | 41.8   | 36.7 ± 2.8 | 79.7      | 76.8      | 75.8         | 35.3 ± 7.4  |

Numbers with ± SD are from three mice and the other numbers are one representative value.
and either Ig H or L chain of 4C8, or mice carrying bcl-2 and IgM incapable of reacting with erythrocytes (Fig. 2 and Table 2). These results indicate that bone marrow B cells are eliminated and failed to generate peripheral B cells in bcl-2 x H x L as well as H x L mice.

To show that maintenance of clonal deletion of self-reactive B cells in the bone marrow in bcl-2 x H x L mice is not due to lack of bcl-2 expression in immature B cells, we sorted pre-B cells (B220+ sIgM-) and B cells (B220+ sIgM+) from the bone marrow of bcl-2 mice (Fig. 3 a) and examined bcl-2 expression in these populations by reverse PCR. Spleenocytes of bcl-2 mice were used as a positive control. The bcl-2 gene was amplified from cDNA of both pre-B and B cells in bcl-2 mice (Fig. 3 b). In contrast, the bcl-2 gene was not amplified in the absence of reverse transcriptase, indicating that genomic DNA was not contaminated in the cDNA samples. Taken together, the bcl-2 gene product does not inhibit clonal deletion of self-reactive immature B cells in the bone marrow.

In bcl-2 x H x L mice, a normal number of Ly-1 B cells (B220+ sIgM-), but only few conventional B cells (B220+ sIgM+) were observed in the peritoneal cavities (Fig. 2 c), showing that conventional but not Ly-1 B cells are deleted in the peritoneal cavity of bcl-2 x H x L mice as is the case for peritoneal B cells in H x L mice (10). The number of conventional B cells in the peritoneal cavity, in contrast, is markedly larger in bcl-2 x HNP x L, bcl-2 x H, bcl-2 x L, and bcl-2 mice than in normal mice. Conventional but not Ly-1 B cells appear to expand in the presence of the bcl-2 gene product when antigen-specific clonal deletion does not take place.

The bcl-2 Gene Product Inhibits Antigen-induced Apoptosis in Peritoneal Ly-1 B Cells. In H x L mice, the intraperitoneal injection of RBCs almost completely eliminates peritoneal Ly-1 B cells by inducing apoptotic cell death (10). We asked if the bcl-2 gene product inhibits the antigen-induced apoptosis of peritoneal Ly-1 B cells by intraperitoneal injection of RBCs. We injected various amounts of RBCs into the peritoneal cavity of bcl-2 x H x L, H x L mice and non-transgenic littermates at 8 wk of age. 12 h after injection, we collected the peritoneal cells and measured the number of peritoneal B cells by two-color staining with the anti-B220 and anti-IgM antibodies, followed by flow cytometry analysis. Although peritoneal Ly-1 B cells were almost completely eliminated in H x L mice with the injection of 10^7 of RBCs, little reduction in the number of peritoneal Ly-1 B cells was observed in both bcl-2 x H x L mice and non-transgenic littermates even with injection of 10^6 RBCs (Fig. 4 a). These results indicate that self-reactive mature B cells were no longer eliminated upon interaction with self-antigens in bcl-2 x H x L mice. Inhibition of clonal deletion of peri-

![Figure 2](image-url)

Figure 2. Flow cytometry analysis of B cells in the H x L, bcl-2 x H x L, bcl-2 x HNP x L, bcl-2 x H, bcl-2 x L, and bcl-2 mice. Cells from the spleen (a), the bone marrow (b), and the peritoneal cavity (c) of transgenic mice at 8 wk of age were collected, washed with PBS twice, and stained with the anti-B220 (6B2) antibody, followed by staining with FITC-labeled mouse anti-rat κ chain antibody and PE-labeled goat anti-mouse IgM antibody. The cells were analyzed by FACScan*, fluorescent four-quadrant, two-color plots were generated with logarithmic amplification of fluorescence emitted by single viable cells. Dots and numbers (%) in the fluorescence windows refer to the cells in the lymphocyte gate as defined by light scatter.
toneal Ly-1 B cells was found in both anemic and nonanemic \textit{bcl-2} \times H \times L mice (data not shown).

To address the question whether \textit{bcl-2} inhibits antigen-induced apoptosis of Ly-1 B cells even by longer antigen exposure, we injected $10^6$ RBCs per day for 7 d intraperitoneally into the \textit{bcl-2} \times H \times L mice, H \times L mice, and nontransgenic littermates. Elimination of peritoneal self-reactive B cells was not observed in \textit{bcl-2} \times H \times L mice, suggesting that \textit{bcl-2} protected the peritoneal B cells from clonal deletion under sustaining exposure to antigens. Furthermore, DNA fragmentation was observed in peritoneal cells of RBC-injected H \times L mice but not in those of \textit{bcl-2} \times H \times L mice nor nontransgenic littermates which received the same treatment (Fig. 5). In summary, these results clearly indicate that the \textit{bcl-2} gene product inhibits antigen-induced apoptosis of peritoneal Ly-1 B cells.

**Discussion**

Elimination of self-reactive B cells is observed in both immature bone marrow B cells and mature peritoneal Ly-1 B cells in H \times L mice (5, 10). By taking advantage of these mice, we assessed the effect of the \textit{bcl-2} gene product on elimination of self-reactive B cells at the immature and mature stage. As is the case for H \times L mice, self-reactive B cells of \textit{bcl-2} \times H \times L mice were eliminated at the stage of bone marrow immature B cells despite \textit{bcl-2} expression in these cells. However, peritoneal Ly-1 B cells were no longer eliminated upon intraperitoneal injection of $10^6$ RBCs for 7 d in \textit{bcl-2} \times H \times L mice, whereas a single injection of $10^7$ RBCs eliminated almost all the peritoneal Ly-1 B cells in H \times L mice. Expression of \textit{bcl-2} in B cells has been shown to increase the number of B cells and the amount of antibody production (23, 24). In \textit{bcl-2} \times H \times L mice, however, neither the number of peritoneal B cells nor antibody production increased as compared with H \times L mice, although antibody production was elevated in anemic mice. Thus, RBC injected into the peritoneal cavity of nonanemic \textit{bcl-2} \times H \times L mice should interact with B cells as effectively as in H \times L mice (10), nonetheless failed to induce apoptosis even when we used 100 times more RBCs required for elimination of B cells in H \times L mice. These results strongly suggest that \textit{bcl-2} blocks antigen-induced apoptosis of peritoneal Ly-1 B cells. Abrogation of apoptosis by \textit{bcl-2} is not restricted to Ly-1 B cells. Indeed, constitutive \textit{bcl-2} expression blocks apoptosis of conventional as well as Ly-1 B cells in peritoneal cavity upon slg cross-linking by anti-Ig injection (Tsubata T., M. Muralcami, and T. Honjo, manuscript submitted for publication). Although it is not clear how many RBCs are required to interact with bone marrow B cells and how long it takes for bone marrow B cells to be eliminated, peritoneal B cells may be exposed to more RBCs for a longer period than B cells by the 7-d consecutive injection of $10^6$ RBCs, which exceeds all the RBCs in the murine bone marrow of a single mouse. This treatment, nonetheless, failed to induce apoptosis of peritoneal Ly-1 B cells in \textit{bcl-2} \times H \times L mice, whereas immature self-reactive B cells in the bone marrow are eliminated in these mice. It is therefore most likely that \textit{bcl-2} has different effects on antigen-induced elimination of bone marrow immature and peritoneal Ly-1 B cells.

In contrast to our results, Hartley et al. (25) demonstrated that the number of immature B cells increased in the bone marrow and spleen in the host mice expressing the hen egg lysozyme (HEL) gene which were reconstituted with bone marrow cells of anti-HEL antibody transgenic mice bearing the \textit{bcl-2} transgene. Different results in the two systems could be due in part to the difference in the expression levels of self-antigens in the bone marrow and the binding affinities between antigens and antibodies. Moreover, irradiated spleen is shown to secrete cytokines that support growth of immature hemopoietic precursors (26). Such a microenvironment may allow expansion of immature B cells expressing \textit{bcl-2} in the spleen as well as bone marrow of irradiated host.
Figure 4. (a) Flow cytometry analysis of peritoneal cells of H × L, bcl-2 × H × L mice and nontransgenic littermates after injection of various amounts of RBC. RBCs (10^9, 10^8, 10^7) in 500 μl PBS were injected intraperitoneously to 8-wk-old mice. After 12 h, mice were killed and peritoneal cells were collected. Cells were subjected to flow cytometry as described in the legend to Fig. 2. Cells from nontreated mice were used as controls. (b) Flow cytometry analysis of peritoneal cells of bcl-2 × H × L mice after injection of 10^9 RBCs per day for seven consecutive days. RBCs (10^9) in 500 μl of PBS were injected intraperitoneously into 12-wk-old bcl-2 × H × L mice (day 0–day 6). On day 7, peritoneal cells of the mice were analyzed using flow cytometry as described above. Peritoneal cells of nontreated bcl-2 × H × L mice at the same age were analyzed in the same way as controls. Percentage of B220^+ IgM^+ B cells is indicated in each compartment.
Figure 5. DNA fragmentation assay of peritoneal cells from RBC-injected transgenic mice. Nontransgenic littermates (lane 1), H x L mice (lane 2), and bcl-2 x H x L mice (lane 3) were injected with 10^8 RBCs. 12 h after injection, peritoneal cells were collected and DNA extracted was electrophoresed in a 2% agarose gel. Note that DNA fragmentation is observed only in DNA from RBC-injected H x L mice.

Although both immature and mature self-reactive B cells have been shown to be clonally deleted by signaling via slg receptors upon interaction with self-antigens (10, 11), mechanisms for antigen-induced elimination of self-reactive B cells appear to be different in immature and mature B cells. This could be due to the fact that signaling pathways via slg receptors are different between the two stages of B cells (11, 12). Different responses to self-antigen stimulation by immature and mature B cells were also shown by recent studies on autoantibody transgenic mice (27, 28) demonstrating that cross-linking of slg induced replacement of the Ig κ chain in self-reactive immature B cells, not in mature B cells. Moreover, maturation arrest but not antigen-induced apoptosis might be involved in clonal deletion of immature B cells (25). Mature B cells are able to respond to foreign antigens, suggesting that stimulation with foreign antigens may activate the machinery rescuing slg-mediated apoptosis of mature B cells. We have shown that signaling through slg does not kill but activates normal B cells in the peritoneal cavity in bcl-2 transgenic mice (Tsubata et al., manuscript submitted for publication). Presumably bcl-2 may play a role in the rescue machinery of mature B cells from slg-mediated apoptosis.

Hemolytic anemia was more frequent and severe in bcl-2 x H x L mice than in H x L mice. Moreover, the severity of hemolytic anemia is proportional to the number of autoantibody producing cells in the peritoneal cavity, in agreement with the finding with H x L mice (10). These findings indicates that peritoneal Ly-1 B cells are responsible for hemolytic anemia in bcl-2 x H x L mice as well as H x L mice. Since the self-reactive B cells in the peritoneal cavity are anyway shielded from the self-antigens (RBCs), it is unlikely that the abrogation of peripheral clonal deletion of self-reactive B cells is responsible for the aggravation of hemolytic anemia in bcl-2 x H x L mice. Rather, prolonged survival for antibody-producing cells might aggravate hemolytic anemia in bcl-2 x H x L mice.

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