Constitutive Expression of Bcl-2 in B Cells Causes a Lethal Form of Lupuslike Autoimmune Disease After Induction of Neonatal Tolerance to H-2b Alloantigens

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Summary

The bcl-2 protooncogene has been shown to provide a survival signal to self-reactive B cells, but it fails to override their developmental arrest after encounter with antigen. Furthermore, constitutive expression of bcl-2 in B cells does not promote the development of autoimmune disease in most strains of mice, indicating that signals other than those conferred by bcl-2 are required for long-term survival and differentiation of self-reactive B cells in vivo. To further examine the factors that are required for the pathogenesis of autoimmune disease, we have assessed the effect of bcl-2 overexpression on the development of host-versus-graft disease, a self-limited model of systemic autoimmune disease. In this model, injection of spleen cells from (C57BL/6 × BALB/c)F1 hybrid mice into BALB/c newborn parental mice induces immunological tolerance to donor tissues and activation of autoreactive F1 donor B cells through interactions provided by alloimmune host CD4+ T cells. BALB/c newborns injected with spleen cells from (C57BL/6 × BALB/c)F1 mice expressing a bcl-2 transgene in B cells developed high levels of pathogenic autoantibodies, but not or barely detectable in mice injected with nontransgenic spleen cells. In mice injected with transgenic B cells, the levels of pathogenic autoantibodies remained high during the course of the study and were associated with increased survival and differentiation of autoreactive B cells, and suggest that both increased B cell survival and T cell help play critical roles in the development of certain forms of systemic autoimmune disease.

The injection of semiallogeneic lymphoid cells into newborn parental mice induces immunological tolerance to donor tissues and establishment of a B and T cell chimerism (1, 2). In addition, the injection of spleen cells differing at MHC class II molecules, but not at MHC class I or non-MHC alloantigens, results in a lupuslike autoimmune syndrome also known as host-versus-graft disease (HVGD)1 (2–5). This syndrome is characterized by hypergammaglobulinemia, predominantly of the IgG1 and IgE isotypes (2), and the production of various autoantibodies (autoAbs), including anti–single-stranded (ss)DNA, antinuclear, and anticytoskeleton autoAbs (2, 6). The autoAb response appears secondary to polyclonal activation of donor B cells by tolerance-resistant alloreactive host Th2 CD4+ T cells (7–10). However, autoAbs with known pathological consequences, such as anti–double-stranded (ds)DNA (11), anti-gp70 (12), antilaminin (13), or these against antineutrophil cytoplasm Ab components (ANCAs) (14, 15), are not found in mice injected at birth with F1 cells. Another important feature of HVGD-associated autoimmune syndrome is that autoAb production is self-limited because of a rapid drop in B cell chimerism by 10 wk of age (2, 16). Indeed, reinjection of spleen cells induces a flare in the serological manifestations (16), emphasizing the central role of donor B cell chimerism in the development and self-limitation of the HVGD in tolerant mice. In addition to autoAb production, limited glomerular lesions are observed in these animals that totally disappear some weeks after the normalization of the serological parameters (16).

The proto-oncogene bcl-2 was the first member of an ex-
Bcl-2 Induces Systemic Lupuslike Disease in Mice Tolerized to Alloantigens

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Materials and Methods

Mice. BALB/c mice were purchased from CRJFFA (Barcelona, Spain). C57BL/6-Ig-bcl-2 transgenic mice (Tg+) were kindly provided by Dr. S.J. Korsmeyer (Washington University, St. Louis, MO; 23). Tg+ or nontransgenic (Tg-) CB6F1 hybrid mice were obtained by crossing BALB/c and C57BL/6-Ig-bcl-2 +/- heterozygous mice. All mice were maintained under pathogen-free conditions.

Induction of Neonatal Tolerance. Neonatal tolerance to H-2 alloantigens was induced by i.p. injection of 0.7 × 10^8 spleen cells from CB6F1-Tg+ hybrid mice or 10^8 spleen cells from CB6F1-Tg- into BALB/c mice within the first 24 h after birth, as described (2). These numbers were chosen in order to inject the same absolute number of B cells in both groups of mice, as determined by flow cytometric analysis. The percentage of plasma cells in donor spleen cells was calculated after Giemsa staining of spleen cell smears.

Flow Cytometric Analysis. CB6F1-Tg+ mice were identified by flow cytometry on PBLs using the 6C8 mAb (20) (hamster anti-human Bcl-2; PharMingen, San Diego, CA) and biotinylated F(ab')2 goat anti-hamster IgG (Jackson ImmunoResearch, West Grove, PA), as previously described (17). To calculate the number of B cells in spleen cell suspensions from CB6F1 mice, cells were stained with FITC-conjugated rat anti-mouse B220 mAb (clone RA3-6B2; PharMingen) and analyzed by flow cytometry. B cell chimerism in spleen and peripheral and mesenteric lymph nodes was assessed by flow cytometry using FITC-anti-B220 mAb and biotin-labeled mouse anti-mouse IAα mAb (clone KH74; PharMingen), followed by streptavidin-PE (Tago, Inc., Burlingame, CA). Cells were analyzed in a FACScan® flow cytometer using Lysys II software (Becton Dickinson & Co., Mountain View, CA). Dead cells and debris were excluded based on forward and sideways light scatter.

Serological Studies. Serum levels of IgG bearing the donor IgGb allotype in tolerant mice were assessed by ELISA as previously described (2). Results were expressed as milligram equivalent per milliliter of IgGb-bearing Ig relative to that detected in the sera of age-matched normal C57BL/6 mice. Serum levels of IgG anti-dsDNA and anti-ssDNA autoAbs were determined by ELISA as described elsewhere (2, 26). IgG anticytoskeleton Abs were evaluated by ELISA as described previously (6). Results of anti-dsDNA, anti-dsDNA, and anti-cytoskeleton Abs were expressed in titration units referred to a standard curve obtained by serial dilutions of a serum pool obtained from 6–8-mo-old MRL +/+ mice. The presence of ANCA was evaluated by indirect immunofluorescence on slides coated with neutrophils (Cormedica, Barcelona, Spain) using a 1/20 dilution of sera followed by FITC-conjugated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark). IgG antiproteinase 3 (anti-PR3) or antitymelyperoxidase (anti-MPO) specificities of ANCA were determined by ELISA. Briefly, 100 μl of diluted serum (1:50) in 2% PBS/0.05% BSA/Tween 20 were incubated for 2 h at room temperature in microtiter wells previously coated with PR3 or MPO antigen (Shield Diagnostics, Dundee, England). After three washes, wells were incubated for 2 h at room temperature with 100 μl of alkaline phosphatase-conjugated goat anti-mouse Ig chain-specific polyclonal Ab (Sigma Chemical Co., St. Louis, MO; 1 μg/ml). Results were expressed in units of absorbance at 405 nm. Serum levels of gp70-anti-gp70 immune complexes (gp70 IC) were determined by ELISA as described previously (27). Results were expressed as nanograms of Ab-bound gp70 per milliliter of serum. Serum RF activity was evaluated by ELISA on microtiter wells coated with mouse-purified mAb of the IgG1 or IgG2a subclasses, as described (27).

Histological Studies. Tissue samples from all major organs were fixed in 10% formaldehyde, imbedded in paraffin, and 3-μm sections were processed for hematoxylin-eosin staining, periodic acid-Schiff staining, and silver-metenamin impregnation, according to standard methods. Glomerular lesions were scored on a 0–4 scale based on the intensity and extent of histopathological changes as described previously (27). Deposition of IgM and IgG subclasses was assessed by direct immunofluorescence on kidney cryocut sections (4 μm) using FITC-rabbit anti-mouse IgM (Cappel Laboratories, Durham, NC) or rabbit anti-mouse IgG subclass-specific antiserum (Southern Biotechnology Associates, Birmingham, AL).

Statistical Analysis. Statistical analysis was performed using the Wilcoxon two-sample test. Probability values >5% were considered insignificant.

Results

Effects of a bcl-2 Transgene on the B Cell Chimerism Associated with Neonatal Injection of F1 Spleen Cells. To analyze whether
Table 1. Evolution of B Cell Chimerism in BALB/c Mice Neonatally Injected with Semiallogeneic Spleen Cells from CB6F1-Tg+ or CB6F1-Tg− Mice

| Cells injected at birth | Percent IAb+, B220+ cells/total B220+ cells |
|------------------------|--------------------------------------------|
|                        | Spleen | Peripheral | Mesenteric |
|                        |        | lymph nodes | lymph nodes |
| CB6F1-Tg−              |        |            |            |
| 5 d                    | 2.3 (0.1)/11.6 | 47.7 (0.6)/55.7 | 37.0 (1.4)/42.7 |
| 3 wk                   | 3.3 (1.5)/30.0 | 2.9 (0.4)/19.6 | 3.0 (0.5)/12.5 |
| 20 wk                  | 1.6 (0.0)/50.0 | 0.9 (0.5)/22.0 | ND |
| CB6F1-Tg+              |        |            |            |
| 5 d                    | 2.8 (1.1)/18.4 | 43.7 (1.3)/57.1 | 27.7 (1.7)/40.1 |
| 3 wk                   | 4.3 (1.4)/33.1 | 6.0 (1.5)/42.9 | 5.4 (1.3)/26.6 |
| 20 wk                  | 20.7 (15.0)/43.2 | 3.0 (2.4)/45.5 | 4.1 (1.6)/36.0 |

Spleen, peripheral, or mesenteric lymph node cells were stained with FITC anti-B220 mAb and biotin-anti-IAb mAb, followed by streptavidin-PE. Results shown indicate the frequency of IAb+ B220+ cells in the total lymphoid population. Mean values and SD (in parentheses) are shown. The frequency of total B220+ cells is also indicated. Results were calculated using five to eight mice in each group.

constitutive expression of bcl-2 in donor B cells influences the development of HVGD in tolerant mice, newborn BALB/c mice (H-2d) were injected intraperitoneally with 0.7 × 10^6 spleen cells from CB6F1-Tg+ or 10^6 spleen cells from CB6F1-Tg− littermates (H-2^b/d) as controls. The number of spleen cells was chosen in order to inject the same absolute number of F1 B cells in both groups of mice since donor T cells but not T cells are essential for HVGD development (2-7, 10). Flow cytometric studies indicated that >98% of the injected splenic B cells from CB6F1-Tg+ and CB6F1-Tg− donors were small IgM+B220+ cells, and <0.5% of the spleen cells from both groups were plasma cells (data not shown). The establishment of tolerance to donor alloantigens was analyzed by measuring the percentage of donor IAb+ cells in spleen and lymph nodes of injected mice. A similar percentage of donor IAb+ cells was observed in spleen and lymph nodes of BALB/c recipients 5 d and 3 wk after injection of either CB6F1-Tg+ or CB6F1-Tg− spleen cells (Table 1). By 20 wk after injection, however, donor IAb+ cells were almost undetectable in BALB/c mice injected with CB6F1-Tg− spleen cells. In contrast, the percentage of donor B cells in BALB/c mice injected with CB6F1-Tg+ spleen cells remained high at 20 wk (Table 1).

To further assess the chimerism and activation of donor B cells, the levels of Ig bearing donor Igbh allotype were analyzed in tolerant mice (2). An increase in the amount of Ig bearing the donor Igbh allotype was observed in both groups of BALB/c mice neonatally injected with CB6F1 spleen cells (Fig. 1). However, the levels of Igs bearing donor Igbh allotype were 5–10 times higher in BALB/c mice injected with CB6F1-Tg+ spleen cells than in the control group injected with CB6F1-Tg− spleen cells. Moreover, BALB/c mice injected with CB6F1-Tg+ cells exhibited Igbh allotype levels that remained elevated at least until week 12 which correlated with a rapid decrease in serum Ig of donor Igbh allotype in mice receiving nontransgenic spleen cells (Fig. 1).

Mice Injected with Splenic B Cells Overexpressing bcl-2 Develop High and Sustained Levels of a Wide Range of AutoAbs. We next examined the production of several autoAbs in BALB/c mice injected at birth with spleen cells from either CB6F1-Tg− or CB6F1-Tg+ mice. As previously reported (2, 28), animals injected with CB6F1-Tg+ spleen cells produced increased levels of IgG anti-ssDNA autoAbs when compared with uninjected or CB6F1-Tg− mice (P < 0.001), but low or undetectable levels of IgG anti-dsDNA autoAbs (Fig. 2A). The kinetics of anti-ssDNA autoAb production in these animals was similar to that described for Igbh production. The levels were maximum at 3–6 wk of age and returned to normal values by 12 wk of age. In contrast, the titers of anti-dsDNA autoAb were two- to threefold higher in BALB/c mice tolerized with CB6F1-Tg+ spleen cells than in the control group and remained elevated during the entire course of the study (P < 0.005 from week 6 to 20) (Fig. 2A). In both groups of animals, IgG anti-ssDNA autoAbs were predominantly of the IgG1 isotype (data not shown), as previously described in this model (2, 8), sug-
gesting that an interaction between host Th2 CD4+ T and donor B cells is also responsible for autoAb production in mice injected with CB6F1-Tg+ spleen cells. Interestingly, the injection of spleen cells from CB6F1-Tg+ mice was accompanied by the production of high levels of IgG anti-dsDNA autoAbs when compared with mice injected with CB6F1-Tg- spleen cells (P < 0.001) (Fig. 2 A).

Since BALB/c mice injected with CB6F1-Tg+ spleen cells produced increased levels of autoAbs, which were undetectable in animals injected with CB6F1-Tg- spleen cells, the presence of a wide spectrum of autoAbs was analyzed in both groups of mice. Abnormal levels of antiaortic myosin, antismooth muscle actin, and antidesmin autoAbs were observed in both groups of tolerized mice, but the levels were much higher in mice injected with CB6F1-Tg- spleen cells (P < 0.01 except for IgG antidesmin autoAbs at weeks 3 and 6) (Fig. 2 B). Significantly, autoAbs reactive with vimentin, keratan sulfate, and laminin were found exclusively in BALB/c mice tolerized with CB6F1-Tg+ spleen cells (P < 0.001) (Fig. 2 B).

Production of ANCA and circulating gp70 IC is associated with accelerated autoimmune disease in humans and mice (12, 14, 15). Indirect immunofluorescence on fixed neutrophils with sera from mice injected with CB6F1-Tg+ spleen cells, but not with sera from animals injected with CB6F1-Tg- spleen cells, showed the typical cytoplasmic and perinuclear staining pattern compatible with the presence of ANCA (data not shown). Significantly, mice neonatally injected with CB6F1-Tg+ spleen cells produced high levels of anti-MPO and anti-PR3 ANCA, detectable by ELISA, at 6 and 12 wk of age (Fig. 3 A). In addition, these animals produced increased levels of gp70 IC (Fig. 3 B). In contrast, animals injected with CB6F1-Tg- spleen cells showed limited or undetectable levels of anti-MPO, anti-PR3 autoAbs and gp70 IC (Fig. 3, A and B). Importantly, un.injected BALB/c mice and donor CB6F1-Tg+ mice failed to produce significant levels of these autoAbs (Figs. 2 and 3), indicating that both the bcl-2 transgene and allogeneic factors are required for the development of a wide spectrum of autoAbs. Finally, we evaluated the presence of anti-IgG1

Figure 2. Serum levels of anti-sDNA, anti-dsDNA (A), and anticytoskeleton (B) autoAbs. BALB/c mice were neonatally injected with spleen cells from CB6F1-Tg+ (●) or from normal CB6F1-Tg− mice (●). Uninjected BALB/c (△) and CB6F1-Tg− mice (○) were used as controls. Results are expressed in titration units by reference to a serum pool from MRL-lpr/lpr mice. Mean ± 1 SD of 15–20 mice per group are represented.
Figure 3. Serum levels of anti-MPO- and anti-PR3 ANCA (A) and nephritogenic anti-gp70 IC (B). (A) BALB/c mice were neonatally injected with spleen cells from CB6F1-Tg + (●) or from normal CB6F1-Tg - mice (○). Uninjected BALB/c (△) and CB6F1-Tg + mice (△) were used as controls. Results are expressed in absorbance units at 405 nm. The production of IgG anti-MPO ANCA between BALB/c mice injected with spleen cells from CB6F1-Tg + and CB6F1-Tg - mice was statistically significant at 12 wk (P <0.001). In contrast, significant differences were observed in the levels of IgG anti-PR3 ANCA between both groups of mice during the entire course of the study (P <0.01). (B) gp70 IC levels were analyzed in 6- and 12-wk-old BALB/c mice neonatally injected with CB6F1-Tg + or with CB6F1-Tg - spleen cells. Results are expressed in nanograms of Ab-bound gp70 per milliliter of serum. The differences between BALB/c mice injected with spleen cells from CB6F1-Tg + and CB6F1-Tg - mice were statistically significant (P <0.01 at 12 wk).

CB6F1-Tg + spleen cells developed a severe form of diffuse proliferative glomerulonephritis characterized by increased mesangial and glomerular cellularity, obliteration of glomerular architecture, and tubular cast formation (Fig. 5, A and C). Immunofluorescence studies demonstrated the presence of IgG and IgM deposits in the glomeruli of these mice (data not shown). In contrast, BALB/c mice injected with CB6F1-Tg - spleen cells exhibited minimal glomerular changes (Fig. 5, B and D). In addition, animals tolerized with CB6F1-Tg + spleen cells developed interstitial lymphocytic pneumonitis characterized by prominent peribronchial (Fig. 5 E) and perivascular (data not shown) accumulation of mononuclear cells, and marked lymphoid infiltrates in the liver resembling chronic active hepatitis (Fig. 5 G). None of these histological abnormalities was observed in BALB/c mice injected with CB6F1-Tg - spleen cells (Fig. 5, F and H). Despite the production of high levels of anti-MPO and anti-PR3 ANCA, BALB/c mice injected with CB6F1-Tg + spleen cells failed to develop typical lesions of vasculitis in small and medium vessels (data not shown).

Discussion

The HVGD that develops in mice tolerized at birth to alloantigens appears to reflect the activation of autoreactive F1 donor B cells by tolerance-resistant host T cells (2, 7–10). We showed in the present report that injection of semiallogeneic splenic B cells expressing a bcl-2 transgene into newborn parental mice induces sustained production of a wide range of autoAbs and early death. The death of these mice appears related to the development of a severe diffuse proliferative glomerulonephritis, although the contribution of liver and lung disease cannot be excluded. This severe form of systemic lupuslike disease markedly contrasts with the mild and self-limited autoimmune reaction that occurs after injection of spleen cells from nontransgenic mice (1, 2, 16).

Experimental work by several investigators has suggested that normal mice contain B cells with autoreactive potential in their peripheral lymphoid repertoire (29–31). However, it is thought that such autoreactive B cells are not efficiently stimulated to produce autoAbs because of the absence
of adequate antigen–specific T cell help (32). In the HVGD model, donor autoreactive B cells are thought to receive stimulatory signals from host helper T cells (7–10). For example, production of autoAbs bearing the allotypic markers of the F₁ donor are not observed in the absence of CD₄⁺ host T cells (7) or after treatment with anti-IL4 mAb (8). The mechanism responsible for the transient course of HVGD appears to be secondary to the progressive loss of B cell chimerism since rejection of F₁ donor B cells into neonatally tolerated animals reactivates the autoimmune manifestations (16). Since bcl-2 confers increased survival to B cells, our findings together with previous results (24, 33) suggest that bcl-2 promotes enhanced and sustained autoAb production by prolonging the life span of autoreactive mature B cells and plasma cells. This conclusion is particularly relevant for B cells that produce certain autoAbs such as anti-DNA, antiantin, antidesmin, or antityrosin autoAbs since they were secreted, although at lower levels, by nontransgenic donor B cells. Thus, these results argue that the limited life span of potentially autoreactive B cells is a major safeguard against the development of certain types of autoAbs and perhaps autoimmune disease. These findings are in accord with those by Strasser et al. (24) who showed that (C57BL/6 × SJL/J)F₁ mice overexpressing bcl-2 in the B cell compartment develop a lethal lupuslike syndrome. However, no such abnormalities were observed in other strains of bcl-2 transgenic mice including C57BL/6, BALB/c, CB6F₁, (C57BL/6 × C3H)F₁, and (SWR/J × SJL/J)F₁ mice (23, 25, the present study, and Merino, R., and G. Nuñez, unpublished results). Together, these observations support the conclusion that signals other than disregulated B cell survival are necessary for disease development. In the current report, we provide evidence that T cell help together with constitutive expression of bcl-2 in B cells are necessary for the production of pathogenic autoAbs and the development of systemic autoimmune disease.

A major observation of the present study was that mice injected with semiallogeneic B cells from CB6F₁-Tg⁺ mice produced high levels of certain pathogenic autoAbs. Indeed, several autoAbs such as those reactive with dsDNA, MPO, PR3, laminin, and gp70 self-antigens have been associated with the development of autoimmune kidney disease in humans and rodents (11–15). These potentially pathogenic autoAbs were almost exclusively found in chimeric mice injected with F₁ B cells expressing the bcl-2 transgene. At least two different possibilities may explain these findings. First, it is possible that spleen cells from normal mice contain B cells or their precursors which, upon injection into parental mice, are capable of producing such pathogenic autoAbs. However, secretion of pathogenic autoAbs might be too low to be detected by the current methodology. In this scenario, bcl-2 promotes the autoAb response and autoimmune disease by increasing the longevity of mature autoreactive B cell clones. A nonexclusive alternative explanation is that production of at least some of the pathogenic autoAbs may be suppressed in normal mice through censoring mechanisms that operate during B cell development. In this context, in vivo models of clonal B cell deletion using transgenic mice expressing Ig receptors specific for hen egg lysozyme (HEL) or surface erythrocyte antigens, have demonstrated that bcl-2 can rescue self-reactive B cells from death during development or in peripheral tissues, respectively (34, 35). However, in the anti-HEL system, self-reactive B cells overexpressing bcl-2 remained arrested and failed to differentiate into mature long-lived B cells (34). In the HVGD model, B cells that produce anti-dsDNA, anti-MPO, anti-PR3, antilaminin, or anti-gp70 autoAb might be rescued from death by bcl-2 overexpression and allowed to expand and mature through signals provided by activated allospecific host CD4⁺ T cells (7–10). Together, our results demonstrate that alteration of programmed cell death regulatory mechanisms in autoreactive B cells in the presence of appropriate T cell help can markedly potentiate the autoimmune process.

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**Figure 5.** Histological analysis of 20-wk-old BALB/c mice injected at birth with spleen cells from CB6F₁-Tg⁺ or CB6F₁-Tg⁻ mice. Representative histological appearance of kidney from mice injected with CB6F₁-Tg⁺ splenocytes showing the presence of a diffuse proliferative glomerulonephritis and tubular cast formation (X30) (A). High power view of glomerulus from mice injected with CB6F₁-Tg⁺ splenocytes showing increased glomerular cellularity and obliteration of glomerular architecture (X300) (C). Peribronchial infiltration of plasma cells in the lung of mice injected with CB6F₁-Tg⁺ spleen cells (X30) (E). Marked lymphoid infiltration of portal tracts extending into the hepatic parenchyma and bridging necrosis in the liver of mice injected with CB6F₁-Tg⁺ spleen cells (X30) (G). Normal histological appearance of kidney (B and D), lung (F), and liver (H) of mice injected with CB6F₁-Tg⁻ spleen cells (X30 and X300 in D).
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