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Immunology

Enforced *BCL2* expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease

(oncogene/transgenic mice/lymphocyte lifespan/lupus mice/glomerulonephritis)

ANDREAS STRASSER, SENGA WHITTINGHAM, DAVID L. VAUX, MARY L. BATH, JERRY M. ADAMS, SUZANNE CORY, AND ALAN W. HARRIS

The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia

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ABSTRACT **The biological functions of the *BCL2* gene were investigated in transgenic mice harboring human *BCL2* cDNA under the control of an immunoglobulin heavy chain enhancer (Eμ). Mice of a representative transgenic strain, Eμ–*bcl*-2-22, had a great excess of B lymphocytes, immunoglobulin-secreting cells, and serum immunoglobulins, attributable to increased longevity of B-lineage cells. Pre-B and plasma cells as well as B cells exhibited prolonged survival in culture. Immunized animals produced an amplified and protracted antibody response. Within the first year of life, most mice spontaneously produced antibodies to nuclear antigens, and 60% developed kidney disease, diagnosed as immune complex glomerulonephritis. Thus Eμ–*bcl*-2-22 mice constitute a transgenic model for a systemic autoimmune disease resembling the human disorder systemic lupus erythematosus.**

The *BCL2* gene was originally identified (1–3) as the locus linked to the immunoglobulin heavy chain locus by the 14;18 chromosome translocation associated with lymphomas of follicular center B-cell origin (4–5). Some cases of chronic lymphocytic leukemia also exhibit a *BCL2* translocation (6). The *BCL2* gene encodes a 24-kDa membrane-associated protein of unknown function, apparently localized to mitochondria (7–9). Its expression is low in normal quiescent B and T cells but increases on mitogenic stimulation (10, 11). Translocation spares the *BCL2* coding region, but the immunoglobulin locus alters its regulation because *BCL2* expression is much higher in B lymphomas bearing a 14;18 translocation than in other types of transformed B cells (10).

BCL2 may be the paradigm of an oncogene that promotes cell survival rather than proliferation. This notion first arose when enforced *BCL2* expression was shown to enhance the survival of interleukin-3-dependent hematopoietic cell lines deprived of growth factor (12, 13). Similarly, it improved the growth of human B- and T-lymphoblastoid cells subjected to various stresses in culture (14–18). Transgenic mice that express a *BCL2* minigene controlled by immunoglobulin regulatory sequences (*bcl*-2–Ig) were found to contain a large excess of B lymphocytes, which showed enhanced survival *in vitro* (19, 20). Our studies on similar mice have confirmed those findings (21) and now extend them by showing effects on pre-B cells and immunoglobulin-secreting cells as well as B cells. Furthermore, we find that these mice develop an exaggerated and prolonged antibody response to immunization and a high incidence of spontaneous systemic autoimmune disease.

MATERIALS AND METHODS

Transgenic Mice. The microinjected transgene construct (21) comprised human *BCL2* residues 1–939 of the cDNA isolated by Cleary *et al.* (22) inserted into the polylinker of the vector pEμSV (23). The Eμ–*bcl*-2-22 transgenic mouse strain was initiated from a (C57BL/6JWehi × SJL/JWehi)F_2 mouse and propagated by serially mating heterozygous transgenic descendants with normal (C57BL/6 × SJL)F_1 hybrids. The second and third generation transgenic descendants used here were identified by dot hybridization of tail DNA with a simian virus 40 probe or by their elevated blood leukocyte counts (21, 24).

Antibodies and Serum Assays. Antibodies and fluorescent conjugates were used in flow cytometric analyses of cell surface antigens as described (24). Cells secreting specific antibody to sheep erythrocytes were quantified by hemolytic plaque counts using the direct technique for IgM (25) and the indirect technique with rabbit anti-IgG antiserum (26) for IgG plus IgM antibody-secreting cells. Spleen cells secreting IgM or IgG of undefined specificity were assayed as hemolytic plaques on protein A-conjugated sheep erythrocytes in the presence of rabbit anti-IgM or anti-IgG antibody (27). Radial immunodiffusion kits for assay of serum immunoglobulins and fluorescein-conjugated antibody to the C3 component of mouse complement were purchased from The Binding Site (Birmingham, U.K.). Fluorescent antibodies specific for mouse IgM, IgG, or κ or λ light chain were purchased from Southern Biotechnology Associates (Birmingham, AL). Fixed HEp-2 cells for detecting anti-nuclear antibodies were obtained from Immuno-Concepts (Sacramento, CA). Antibodies to double-stranded DNA were detected by using nuclease-treated ^{14}C-labeled *Escherichia coli* DNA (Amersham). Calf thymus histones were obtained from Cooper Biomedical; rabbit thymus extract was obtained from Pel-Freez Biologicals. Serum urea assays employed a urease conductivity rate method in a CX3 analyzer (Beckman).

RESULTS

Prolonged Survival of B-Lineage Cells *in Vitro*. Of the 18 *bcl*-2 transgenic strains we developed, 10 exhibited abnormalities in the B-lymphoid lineage (21); one of these, Eμ–*bcl*-2-22, was selected for detailed analysis. In the spleen and activated B cells of these mice, transcripts of the transgene were 20- to 50-fold more abundant than those of the endogenous *Bcl-2* gene, while little if any transgene expression was detectable in T cells (data not shown). Consistent with this expression pattern, B-lymphoid cells from hematopoietic tissues of Eμ–*bcl*-2-22 mice exhibited strikingly prolonged survival in culture, but neither thymocytes nor peripheral T cells were affected. In cultures from lymph nodes, spleen, and peripheral blood, essentially all the surviving cells were small B cells (21). The survival advantage is not, however, confined to this stage of B-lymphoid development. In 2-week cultures of bone marrow, 25% of the survivors were pre-B

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Abbreviation: Sm/RNP, Sm/ribonucleoprotein antigen.

cells (Fig. 1*A*). Plasma cells also appear to survive longer, since specific antibody-secreting cells in spleen cell cultures from immunized transgenic mice persisted for at least 3 weeks, whereas those from immunized nontransgenic littermates were undetectable after 6 days (Fig. 1*B*). Similarly, the transgene prolonged the functional life of IgM-secreting cells generated by treating spleen cells *in vitro* with the B-cell mitogen lipopolysaccharide (data not shown).

Accumulation *in Vivo* of Small B Cells, Immunoglobulin-Secreting Cells, and Serum Immunoglobulin. The most conspicuous abnormality in Eμ–*bcl*-2-22 mice was their excess of small B lymphocytes. From 3 weeks of age, the animals showed a 3- to 7-fold increase in circulating leukocytes, 80–90% of which were surface immunoglobulin-bearing B cells. On average, the spleen of a 6- to 14-week-old Eμ–*bcl*-2-22 mouse contained 3×10^8 lymphocytes instead of the normal 1×10^8. Most of the increase was due to B lymphocytes, which numbered 1.5×10^8–2.5×10^8 rather than the usual 5×10^7, whereas T-cell numbers were unaltered. The massive follicular hyperplasia reported as a feature of *bcl*-2–Ig mice (19) was not evident in young healthy Eμ–*bcl*-2-22 mice; instead, the excess B cells were distributed through both follicular and nonfollicular zones. Excess B cells were also evident in the bone marrow and lymph nodes (Table 1) and the peritoneal cavity (data not shown) but not in the thymus, which contained normal numbers of the four major subpopulations of thymic lymphocytes defined by the surface markers CD4 and CD8 (data not shown). Pre-B-cell numbers were also normal (Table 1).

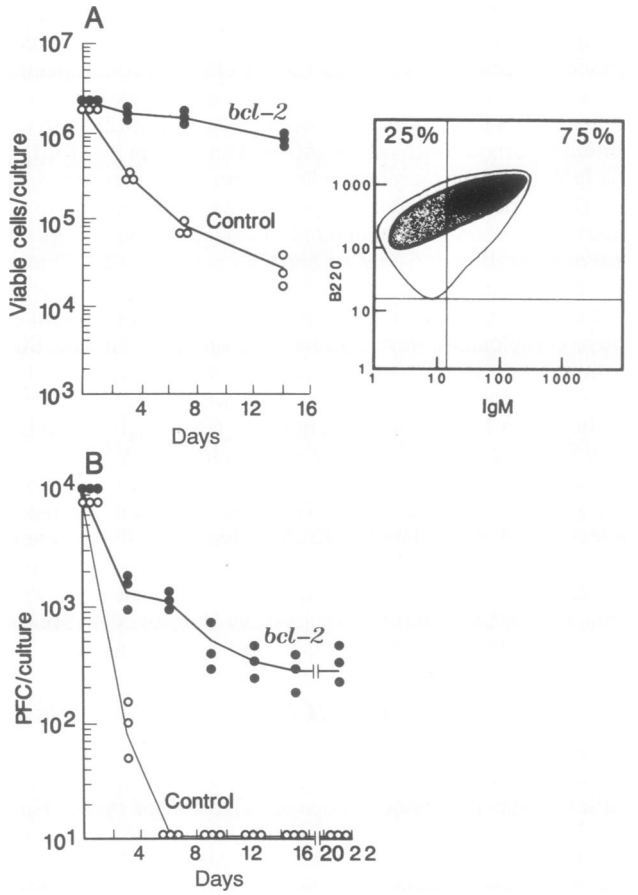

FIG. 1. Prolonged survival of pre-B, B, and plasma cells from Eμ–*bcl*-2-22 (*bcl*-2) mice. (*A Left*) Bone marrow cells were assayed for viability by trypan blue exclusion. (*A Right*) After a 14-day incubation, the cells were assayed by dual-color immunofluorescence and flow cytometry for B cells (B220-positive, IgM-positive) and pre-B cells (B220-positive, IgM-negative). The pre-B cells were confirmed to be surface immunoglobulin heavy and light chain-negative (data not shown). (*B*) Splenic IgM anti-sheep erythrocyte antibody-forming cells from mice immunized with sheep erythrocytes 5–10 days previously were enumerated by hemolytic plaque assay (25). The culture medium was Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μM asparagine, and 50 μM 2-mercaptoethanol. PFC, plaque-forming cells.

The expanded population of Eμ–*bcl*-2-22 B cells expressed normal levels of IgM, IgD, CD45R (B220), and class II major histocompatibility complex antigens (21) and did not express the Ly-1 or Mac-1 markers of the CD5 B-cell sublineage (data not shown). Flow cytometric measurement of their light-scattering capacity showed that most were small (21), like normal quiescent B cells. Thus the transgene did not drive B cells to proliferate but caused them to accumulate, apparently by prolonging their life span.

Immunoglobulin-secreting cells, the terminally differentiated descendants of stimulated B cells, also accumulated in Eμ–*bcl*-2-22 mice. The animals had a 3- to 10-fold excess of IgM-secreting cells in the spleen and a 50- to 200-fold excess of IgG-secreting cells (Fig. 2). This was accompanied by a marked increase in serum immunoglobulins (Table 2). By 3 months of age, mean IgM levels were elevated 2-fold, IgG levels were elevated 5-fold, and IgA levels were elevated 7- to 8-fold above the levels in nontransgenic littermates.

Amplified and Prolonged Immune Response. To explore how enforced *BCL2* expression affects a well-studied humoral immune response, we immunized Eμ–*bcl*-2-22 mice with sheep erythrocytes and enumerated splenic IgM and IgG hemolytic plaque-forming cells at intervals thereafter. The IgM response per spleen was almost 10-fold higher than that in nontransgenic animals (Fig. 3*A Upper*), although its peak (at 7 days) was somewhat delayed. The IgG response was elevated 5-fold (Fig. 3*A Lower*). Presumably the increased amplitude of the response reflects, at least in part, the increased number of splenic B cells.

The most striking alteration in the immune response was, however, its persistence. After a single immunization, plateau levels of IgM and IgG antibody-forming cells were maintained for at least 6 weeks, whereas those in control animals returned to basal levels in 3 weeks (Fig. 3*A*). We attribute the remarkably sustained immune response to an extended life span of plasma cells, a property consistent with the enhanced survival of antibody-secreting cells in tissue culture (Fig. 1*B*). A second immunization did not further

Table 1. Cellular composition of hematopoietic and lymphoid organs in healthy Eμ–*bcl*-2-22 (*bcl*-2) and control mice

Organ	Composition, % of total nucleated cells			
	Pre-B	B	T	Myeloid
Spleen				
bcl-2	<2	72 ± 4	14 ± 4	4 ± 1
Control	<2	50 ± 4	32 ± 3	6 ± 2
Bone marrow				
bcl-2	20 ± 4	43 ± 9	3 ± 1	14 ± 5
Control	18 ± 6	9 ± 2	5 ± 1	38 ± 2
Lymph node				
bcl-2	<2	49 ± 4	44 ± 5	<2
Control	<2	28 ± 2	68 ± 3	<2

The data (mean ± SD) were derived from four to six healthy transgenic mice and their nontransgenic littermates at 6–12 weeks of age. Cells were stained with fluorochrome-labeled monoclonal antibodies and quantified by flow cytometry (24). Pre-B cells were CD45R (B220)-positive, IgM-negative (also immunoglobulin light and heavy chain-negative); B cells were CD45R (B220)-positive, IgM-positive; T cells were Thy1-positive, $\alpha\beta$ T-cell receptor-positive; myeloid cells were Mac-1-positive. Spleens from Eμ–*bcl*-2-22 mice contained on average three times more leukocytes than those from nontransgenic littermates (Control), but the cellularity of bone marrow and lymph nodes was essentially normal.

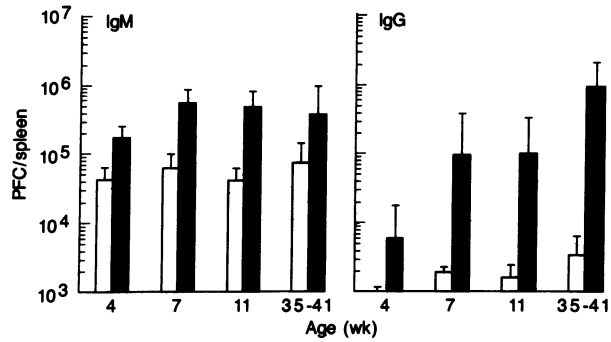

FIG. 2. Increased immunoglobulin-secreting cells in Eμ–*bcl*-2-22 mice. Spleen cell suspensions from three to five transgenic (filled bars) and nontransgenic (open bars) littermates at each indicated age were assayed for hemolytic plaque-forming cells (PFC) by the protein A technique (27). The results are presented as geometric means with upper limits of standard deviations.

boost the number of antigen-specific IgM- or IgG-secreting cells in the transgenic mice (Fig. 3*B*), perhaps indicating that the antigen was rapidly neutralized by antibody persisting from the primary response.

Systemic Autoimmune Disease. Between 3 and 12 months of age, 60% of the Eμ–*bcl*-2-22 mice became terminally ill (Fig. 4), displaying hunched posture, ruffled fur, weight loss, and edema. Autopsy typically revealed enlarged lymph nodes (5- to 20-fold) and spleen (2.4- to 15-fold), due primarily to an increase in small and medium-sized B lymphocytes. These lymphoid accumulations were not neoplastic, since Southern analysis (13 mice) with an *Igh* joining region probe provided no evidence of clonal restriction, and transplantation from 15 donors yielded no tumors in 6 months of observation.

Although 30% of sick Eμ–*bcl*-2-22 mice exhibited histologic evidence of myocardial infarction, the cause of terminal illness in most cases (87%) appeared to be renal failure. The kidneys were pale and uneven in texture, and terminal serum samples contained urea levels 2- to 15-fold higher than the mean (9.0 ± 2.4 mM) in healthy nontransgenic littermates, indicating extensive loss of renal glomerular function. Histologic examination revealed severe glomerular abnormalities and dilated renal tubules containing amorphous eosinophilic casts (Fig. 5 *A* and *B*). As in some other organs (liver, lung, and salivary glands), nodular noninvasive masses of lymphocytes and plasma cells surrounded some arteries (Fig. 5*A*). Fluorescent antibodies against mouse immunoglobulin stained the casts in the kidney tubules and produced strong, granular staining (Fig. 5*C*) of essentially all glomeruli in all nine cases tested (Table 3). IgM and IgG containing both κ and λ light chains were detected by immunofluorescence, and antibody against the C3 component of mouse complement also stained the glomeruli but not the tubular casts (data not shown). The kidney lesions were therefore diagnosed as immune complex glomerulonephritis.

Healthy one-year-old transgenic animals had immunoglobulin and complement deposits in most glomeruli as well as lymphoid nodules around some renal arteries, but usually no other histologic signs of kidney disease. Overt kidney disease might have ensued had these mice been allowed to live longer, as the cumulative incidence was still rising at one year (Fig. 4) when monitoring was terminated. No kidney disease was seen in nontransgenic littermates, and at one year of age they showed only sparse deposits of immunoglobulin in some glomeruli (Table 3).

Table 2. Increased serum immunoglobulins in healthy Eμ–*bcl*-2-22 (*bcl*-2) mice

Mice (*n*)	Age, mo	Serum immunoglobulin, mg/ml		
		IgM	IgG	IgA
Control (9)	3–9	0.22 ± 0.04	5.4 ± 0.6	1.2 ± 0.2
bcl-2 (6)	3	0.45 ± 0.08	21 ± 5	9.6 ± 4.9
bcl-2 (10)	6	0.32 ± 0.05	31 ± 7	8.1 ± 2.2
bcl-2 (4)	12	0.79 ± 0.19	25 ± 5	11 ± 5

Immunoglobulin levels measured by radial immunodiffusion are shown as mean values ± SEM. Control mice were nontransgenic littermates.

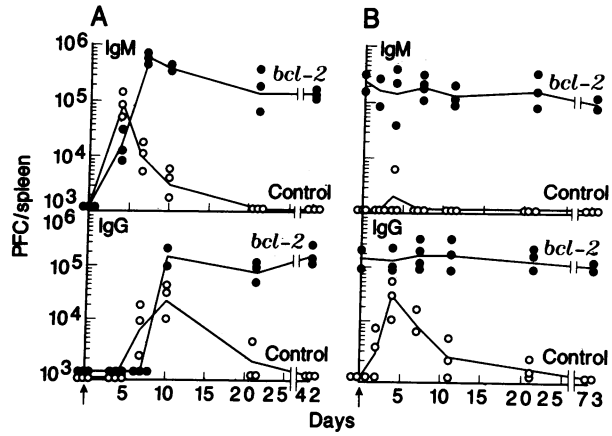

FIG. 3. Increased and prolonged immune response in Eμ–*bcl*-2-22 (*bcl*-2) mice. IgM and IgG antibody-secreting cells generated by primary (*A*) and secondary (*B*) responses to antigen were enumerated. Mice were immunized with an intraperitoneal injection of 0.1 ml of 1% (vol/vol) sheep erythrocytes in saline. Some received a second immunization 28 days later. At the indicated times, three mice per group were sacrificed and the antibody-secreting cells among dispersed spleen cells were assayed. The direct hemolytic plaque technique was used for IgM assays (25), and the indirect technique (26) was used to enumerate IgM- plus IgG-secreting cells.

Immune complex glomerulonephritis was suggestive of an autoimmune etiology. Consistent with this possibility was the presence of high-titer anti-nuclear antibody in all of the sick animals and in most of the transgenic mice that had not developed disease by one year of age, but in very few year-old control mice (Table 3). Serum from all nine sick mice tested gave homogeneous or speckled staining of the test nuclei in immunofluorescence assays. All contained antibodies against histones and two also had antibodies to double-stranded DNA. Significantly, the two that produced speckled staining of nuclei contained antibodies against Sm/ribonucleoprotein (Sm/RNP) antigens (Table 3). In humans, this activity is uniquely, although not uniformly, associated with the autoimmune disease systemic lupus erythematosus (30). Although one mouse had antibodies to cardiolipin and one had rheumatoid factor, their frequency was no greater

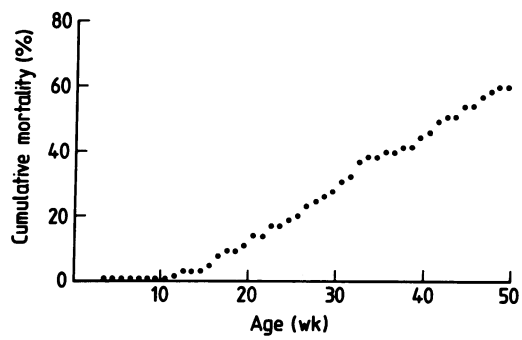

FIG. 4. Cumulative incidence of disease with age among Eμ–*bcl*-2-22 mice. Individuals among a group of untreated transgenic animals were killed when they showed general signs of illness (see text). Data from 31 females and 34 males are pooled as no difference in disease incidence was apparent.

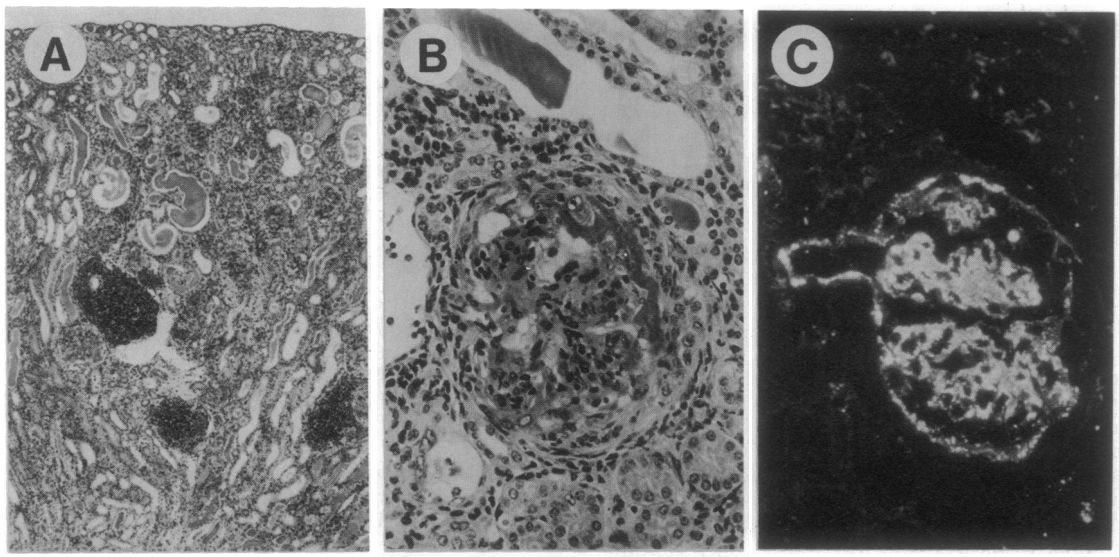

FIG. 5. Kidney pathology in terminally ill Eμ–*bcl*-2-22 mice. (*A*) Histologic section at low power, showing dense periarterial deposits of lymphoid cells and dilated tubules containing casts. (×40.) (*B*) High-power view of a glomerulus, showing hypercellularity, increased mesangial matrix and decreased functional capillary loops, deposition of eosinophilic material (possibly exudation of fibrin), and proliferation of capsular epithelial cells to form a "crescent" (×200.) (*C*) Fluorescence microscopic image of a frozen section stained with fluorescein-conjugated sheep antibody against mouse immunoglobulin (Silenus Laboratories, Dandenong, Australia), showing a strong, granular reaction in the glomerulus and its capsule (×200.) The histologic sections were cut from paraffin-embedded, Bouin's-fixed tissues and stained with hematoxylin/eosin.

than that in large groups of nontransgenic littermates. Tests for a range of autoantibodies associated with various other autoimmune diseases were negative. These included antibodies to mitochondria, smooth muscle, glomerular basement membrane, renal tubules, thyroid, and gastric antigens.

DISCUSSION

Our analysis of transgenic mice supports the hypothesis that the *BCL2* oncogene acts within a cell "survival" signal pathway (12). *In vitro*, the survival advantage conferred by the Eμ–*bcl*-2 transgene is exhibited by B cells, as also observed by McDonnell *et al.* (19, 20), and also by pre-B cells (Fig. 1*A*) and plasma cells (Fig. 1*B*). In the whole animal, it causes a notable perturbation of lymphoid homeostasis. The Eμ–*bcl*-2-22 mice display a striking excess of small, nonneoplastic B cells (Table 1), an increased number of immunoglobulin-secreting plasma cells (Fig. 2), and high levels of circulating immunoglobulin (Table 2). Significantly, the mice display a heightened and prolonged antibody-forming cell response to immunization (Fig. 3), a finding that may prove of practical use, for example, in obtaining monoclonal antibodies to rare specificities. All these findings are consistent with the notion that constitutive *BCL2* expression interferes with mechanisms ensuring the normal turnover of B-lymphoid cells.

Table 3. Autoantibodies and kidney abnormalities in Eμ–*bcl*-2-22 mice

Antibody or feature	Control	Transgenic	
		Healthy	Ill
Anti-cell nuclei*	3	6	9
Anti-dsDNA	0	2	2
Anti-histone	2	5	9
Anti-Sm/RNP	0	1	2
Glomerular Ig†	13	7	9
Tubular casts	0	0	9
Lymphoid foci	0	5	9

The number of mice positive for each indicated test is shown. Healthy mice ($n = 13$ control mice and $n = 7$ healthy transgenic mice) were tested at 12 months of age, and sick mice ($n = 9$) were tested at autopsy (20–44 weeks). Anti-nuclear antibodies were detected by immunofluorescence on HEp-2 cells (28); anti-histone antibodies were detected by immunoblotting tests on calf thymus histones; antibodies to double-stranded DNA (dsDNA) were determined by a modified Farr immunoassay; antibodies to Sm/RNP were determined by counterimmunoelectrophoresis using mixed nuclear extracts of rabbit thymus and human spleen (29) and by immunoblotting using an electrophoretically fractionated extract of HeLa cell nuclei (28). Glomerular deposits of immunoglobulin (Ig) were detected in frozen sections of kidney by direct immunofluorescence (28). Tubular casts and perivascular foci of lymphoid cells in the kidney were observed histologically.

*Ranges of titers from positive sera were 10^2–10^3 for healthy control mice, 10^2–10^4 for healthy transgenic mice, and 10^2–2×10^4 for ill transgenic mice (negative sera gave no reaction at a 1:10 dilution).

†Ranges of percentages of renal glomeruli containing immunoglobulin were 1–50% for healthy control mice, 50–80% for healthy transgenic mice, and 90–100% for ill transgenic mice.

Unexpectedly, by one year of age, the majority (60%) of Eμ–*bcl*-2-22 mice suffered a terminal disorder with several features of autoimmunity (Table 3). Antibodies to nuclear components were prevalent, most frequently to histones but also to double-stranded DNA and the Sm/RNP antigen, two hallmark activities in the serum of certain patients with systemic lupus erythematosus (30). In the sick animals, blood urea levels were increased, and the renal glomeruli were structurally abnormal and contained immune complexes (Fig. 5). It is therefore likely that immune complex glomerulonephritis precipitated the terminal illness. This autoimmune syndrome was a common occurrence in independent Eμ–*bcl*-2 transgenic strains that we have developed (ref. 21; unpublished observations) and therefore is not determined by the transgene insertion site. On the other hand, the *bcl*-2–Ig transgenic mice of McDonnell *et al.* (19, 20) have not been reported to develop renal disease. One difference between those mice and the Eμ–*bcl*-2 mice described here is their genetic background: C57BL/6 × C3H in the former and C57BL/6 × SJL in the latter. Although we have not seen any substantial renal disease in nontransgenic littermates, the SJL component in our mice may provide a genetic environment that favors the development of autoimmunity.

Cells secreting autoantibodies occur in normal mice (e.g., refs. 31 and 32), but their number is probably limited by the normal turnover process that limits their life span to a few

days. We suggest that continuous expression of the *BCL2* transgene increases the longevity of these cells and their autoreactive B-cell precursors and that their accumulation eventually allows the autoantibodies to reach pathogenic levels. Although we cannot exclude the possibility that a transgene-induced abnormality of T-cell function contributes to the development of the autoimmunity, the strain studied here evinces no evidence of T-cell accumulation *in vivo* or of prolonged survival of T cells *in vitro*.

The Eμ-*bcl*-2-22 mouse represents a transgenic model of systemic autoimmune disease. Several of its features, including immune complex glomerulonephritis, anti-nuclear antibodies, lymphadenopathy, elevated immunoglobulins, and myocardial infarction, are shared with previously established mouse strains that have been extensively studied as models of human systemic lupus erythematosus (reviewed in ref. 33). These strains—(NZB × NZW)F_1, BXSB, and MRL/Mp-*lpr*/*lpr*—appear to depend on several poorly understood genetic loci for development of disease (33). In the transgenic model, a single dysregulated gene, *BCL2*, provokes systemic autoimmunity, apparently by extending the functional lifespan of B lymphocytes and plasma cells. This implies that the rapid turnover of plasma cells in the spleen and lymph nodes of the normal mouse is a significant component of the barrier against the development of pathologic autoimmunity.

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