Reduced Life Span of Anergic Self-reactive B Cells in a Double-transgenic Model

By David A. Fulcher and Antony Basten

From the Centenary Institute of Cancer Medicine and Cell Biology, University of Sydney, Newtown, New South Wales 2006, Australia

Summary

The life span of anergic self-reactive B cells was determined by 5-bromo-2'-deoxyuridine (BrdU) loading of tolerant double-transgenic (Dbl-Tg) mice produced by mating hen egg lysozyme (HEL)-transgenic mice with the corresponding immunoglobulin-transgenic (Ig-Tg) mice, the B cells of which express anti-HEL IgM and IgD. B cells from Dbl-Tg mice, despite being exposed to soluble antigen throughout their development, are not deleted, but persist in an anergic state. As a prelude to studying the life span of these anergic B cells, BrdU was administered to nontransgenic mice; B cells from the bone marrow, spleen, and lymph nodes displayed distinct kinetic profiles based on reciprocal expression of the B220 isoform of CD45 and heat-stable antigen (HSA). Thus, immature $B220^{lo}/HSA^{hi}$ B cells incorporated BrdU rapidly suggesting recent generation from dividing precursors, whereas uptake by B cells expressing the mature $B220^{hi}/HSA^{lo}$ phenotype was significantly slower, consistent with a longer life span. Such gating allowed analysis to be directed at the stable mature B cell population in transgenic mice. Comparison of BrdU uptake in Ig- and Dbl-Tg mice indicated that B cells from Dbl-Tg mice were renewed at a much higher rate (50% renewal times of 0.64 vs. 3.4 wk for total B cells, and 1.2 vs. 5.0 wk for mature $B200^{hi}/HSA^{lo}$ cells from Dbl- and Ig-transgenic mice, respectively). This difference was even more marked when analysis in Dbl-Tg mice was restricted to HEL-binding cells, which had a 50% renewal time of 3–4 d compared with 4–5 wk for non-HEL-binding B cells. While the proportion of B cells in cell cycle, and the rate of entry of newly generated B cells into the spleen of Ig- and Dbl-Tg mice, were similar, B cell numbers were reduced in Dbl-Tg mice. It was therefore concluded that anergic B cells have a markedly decreased life span in the periphery. According to studies in radiation chimeras produced by reconstituting HEL-transgenic recipients expressing different serum levels of antigen with Ig-Tg bone marrow, the reduced life span of anergic B cells was associated with the anergic state per se, the serum concentration of HEL being important only in attaining the critical threshold necessary for tolerance induction. B cells rendered tolerant by exposure to soluble self-antigen therefore survive for a relatively short period in an anergic state once they have reached peripheral lymphoid tissue and fail to enter the long-lived compartment. These findings have implications for the maintenance of self-tolerance and induction of autoimmunity in both B cell and T cell repertoires and suggest that the distinction between B cell anergy and deletion is more relative than absolute.

Studies in double-transgenic (Dbl-Tg)[1] mice containing endogenously expressed antigen and monoclonal populations of T cells and B cells of the same specificity have confirmed that the predominant mechanisms responsible for self-tolerance are clonal deletion and anergy (1–5). In our hen egg lysozyme (HEL)/anti-HEL transgenic model of self-tolerance, the fate of autoreactive B cells was shown to be determined predominantly by the structure of the neoself-antigen (6). When mice transgenic for anti-HEL IgM and IgD (Ig-Tg) were mated with HEL transgenic mice expressing the antigen in membrane bound form (mHEL-Tg), the Dbl-Tg offspring were tolerant, and very few B cells expressing transgene-encoded Ig were detected in peripheral lymphoid tissue (7). This proved to be the result of a block in B cell differentiation at an immature stage within the bone marrow; loading studies using 5-bromo-2'-deoxyuridine (BrdU) showed that these cells had an average life span of ~15 h (8). By contrast, B cells exposed to HEL in soluble form (sHEL) were not deleted from peripheral lymphoid tissue; rather, a significant proportion of them appeared to migrate normally from bone marrow to spleen where they colonized the B cell

[1] *Abbreviations used in this paper:* BrdU, 5-bromo-2'-deoxyuridine; Dbl-Tg, double-transgenic; HEL, hen egg lysozyme; HSA, heat-stable antigen.

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follicles in an anergic state, displaying selective downregulation of IgM but not IgD antigen receptors (9).

The apparent persistence of potentially self-reactive B cells in spleen and lymph node poses questions about their role both in maintenance of self-tolerance and induction of autoimmunity. However meaningful answers depend on knowing the renewal rate and longevity of these B cells once they have been rendered anergic.

The aim of this paper was to compare the life span of transgene-encoded B cells from tolerant sHEL-Dbl-Tg vs. Ig-Tg mice by continuous loading with BrdU. Mature peripheral B cells defined by the $B220^{hi}HSA^{lo}$ phenotype in the spleen and bone marrow of nontolerant Ig-Tg controls had a 50% renewal rate of 5–6 wk whereas this figure was greatly reduced for anergic B cells from Dbl-Tg mice, being as low as 3–4 d when analysis was confined to HEL-binding cells. These findings challenge the dogma that deletion and anergy are completely distinct processes and point to a single fate for B cells rendered tolerant by interaction with self-antigen in the absence of costimulatory signals.

Materials and Methods

Mice. Conventional inbred C57BL/6 (B6), SJL/J, and (B6 × SJL/J)F_1 strains of mice and transgenic mouse lines were housed under standard conditions in the University of Sydney animal facility. Hemizygous Ig and HEL transgenic lines were maintained on a B6 or (B6 × SJL/J)F_1 background by backcrosses with nontransgenic mice. The Ig-Tg mice belonged to the MD4 line, the B cells from which expressed high affinity IgM and IgD receptors for HEL (10). In the HEL-Tg mice, HEL was expressed in soluble form under control of the mouse metallothionein-1 promoter (lines ML4, ML5) or the albumin promoter (line AL3) (11). Soluble HEL-Dbl-Tg mice were created by mating ML5 HEL-Tg with MD4 Ig-Tg mice.

Cell Suspensions. Mice were sacrificed by cervical dislocation, the spleen, or inguinal lymph nodes were dissected out, gently pressed through a sieve using a syringe plunger, and suspended in PBS supplemented with protein (0.1% BSA or 2% FCS; Commonwealth Serum Laboratories, Victoria, Australia) and 0.1% sodium azide. Bone marrow suspensions were prepared by flushing femurs in both directions with the same medium.

Bone Marrow Chimeras. Non-Tg or sHEL-Tg mice (from ML4, ML5, or AL3 lines), aged at least 3 mo, were lethally irradiated (950 rad) and reconstituted by intravenous injection of 2.5×10^6 bone marrow cells from MD4 Ig-Tg donors. The resultant chimeras were used at least 9 wk after reconstitution.

Monoclonal Antibodies. The following mAbs were used (specificities in parentheses): HyHEL5 (HEL [12]); RA3-6B2 (B220 [13]); M1/69 (heat-stable antigen [HSA] [14]); and BU-44 (BrdU [15]). Details on fluorochrome conjugates are given in the subsequent sections. Aggregates from FITC-conjugated and biotinylated mAbs were removed before use by centrifugation in an Airfuge™ (Beckman Instruments, Palo Alto, CA) at 28 psi for 15 min.

Administration of BrdU. BrdU (Sigma Chemical Co., St. Louis, MO) was administered to mice in the drinking water at a concentration of 0.25 mg/ml for 1–6 wk. This dose was selected on the basis of preliminary experiments that indicated that it resulted in sufficient DNA incorporation to permit ready discrimination between BrdU-positive and -negative cells in the absence of significant bone marrow toxicity. Water bottles containing BrdU were shielded from the light and changed twice weekly.

BrdU Assay. The method used to detect BrdU in B cell DNA was as reported previously (8). Briefly, suspensions of bone marrow and spleen cells were labeled with biotinylated anti-HSA antibody. After fixing in 0.5% paraformaldehyde in PBS (20 min), DNA was denatured using 3-N HCl supplemented with 0.5% Tween 20 to permeabolize the cell membrane (20 min). The acid was then neutralized with 0.1 M disodium tetraborate (3 min) and the cells were stained with FITC-conjugated anti-BrdU and PE-conjugated anti-B220 antibodies. The biotinylated antibody was developed with streptavidin conjugated to Tricolor™ (Caltag, South San Francisco, CA) or Quantum Red (Sigma Chemical Co.). Although Tricolor™ and Quantum Red bind to HEL nonspecifically on fresh cells (our unpublished observations), this did not appear to occur after the fixation and denaturation procedure used here. The HEL-binding assay was performed in an identical fashion to the above except that the anti-HSA staining step was replaced by a sandwich stain involving incubation with HEL followed by biotinylated HyHEL5. Listmode data on $1.0–1.5 \times 10^4$ lymphocyte events were collected. Analysis gates for BrdU incorporation were set between two clearly separate populations, the negative population corresponding to that obtained in control mice not fed BrdU.

FACS® Analysis. Cell suspensions stained as described previously were subjected to three-color analysis on a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA) which collected data on forward and orthogonal light scatter and fluorescence intensity. Compensation for spectral overlap was based on the fluorescent signal from singly labeled cells. Listmode data were collected and analyzed using LYSIS 2.0 software on a Hewlett-Packard computer.

Cell Cycle Analysis. 10^6 spleen or bone marrow cells were labeled either with anti-B220 directly conjugated to FITC, or HEL (200 ng/ml, Sigma Chemical Co.) followed by FITC-conjugated HyHEL5. Cells were fixed by the dropwise addition of ice-cold 70% ethanol and kept at 4°C for up to 1 wk. They were then resuspended in PBS with 1% glucose, 50 μg/ml propidium iodide (Sigma Chemical Co.), and 80 ng/ml RNase, DNase-free (Boehringer-Mannheim, Mannheim, Germany). Stained cells were analyzed by two-color flow cytometry with propidium iodide fluorescence being detected by linear amplification of the signal from the second fluorescent channel. Listmode data on $3–5 \times 10^4$ events were collected; doublets were removed from analysis using the doublet-discrimination module and singlet gating based on the width of the signal from the second fluorescent channel. DNA content was determined by the height of this signal after gating on cells exhibiting a lymphocyte scatter profile that also expressed B220 or bound HEL.

Analysis of BrdU Uptake Patterns. The pattern of appearance of BrdU-positive cells within a defined population during loading studies is dependent on its kinetic makeup. A population that is slowly but continually renewed from precursors will show a smooth gradual uptake pattern the slope of which is dependent on the average life span of that population (single-order kinetics). Alternatively, a cell population may contain a combination of short- and long-lived cells; in this case a rapid increase in labeling occurs until almost 100% of the short-lived population has incorporated BrdU, followed by a more gradual increase, representing the slower renewal of the longer-lived population. Consequently the BrdU labeling data are presented in terms of the time taken to label 50% of the population, referred to subsequently as the 50% renewal time. To calculate this, BrdU uptake data were plotted, a line of best fit was drawn, and the time to 50% labeling was estimated. While such

a mode of analysis makes no assumptions as to the actual B cell kinetics, in many cases the appearance of BrdU-labeled cells in a first-order kinetic population was found to follow a pattern that could best be described by the exponential decay of the unlabeled population. Based on this assumption, the 50% renewal time is equivalent to the population half-life.

Results

Expression of B220 and HSA Defines Distinct Kinetic Populations in Central and Peripheral Lymphoid Tissue. The various stages in B cell maturation can be defined by reciprocal changes in expression of the two surface markers, B220 and HSA (16, 17). Thus, pre-B and immature B cells express the $B220^{lo}/HSA^{hi}$ phenotype whereas mature B cells are $B220^{hi}/HSA^{lo}$. Initially, BrdU loading was carried out in non-Tg mice to determine whether the two B cell populations defined by B220 and HSA expression had distinct kinetic profiles. Non-Tg B6 or (B6 × SJL/J)F_1 mice were given BrdU for 7 d and uptake was assessed in B cells obtained from the bone marrow, spleen, and lymph nodes by three-color FACS® analysis. As shown in Fig. 1 *A*, good discrimination between immature $B220^{lo}/HSA^{hi}$ and mature $B220^{hi}/HSA^{lo}$ B cells was obtained. In the bone marrow, BrdU-positive B cells accumulated rapidly, 61% being labeled over the 7-d loading period (Fig. 2). This figure rose to almost 100% when uptake was assessed in the immature $B220^{lo}/HSA^{hi}$ subset, which made up ~29% of bone marrow lymphocytes (Fig. 1 and Table 1). On the other hand, B cells expressing the mature $B220^{hi}/HSA^{lo}$ phenotype took up BrdU more slowly, so that only 11% were labeled over the same time period (Fig. 2). These cells represented IgD-positive B cells that had returned from the periphery to the bone marrow (18) where they comprised about a quarter of the lymphocytes (Table 1).

Analysis of the BrdU uptake pattern of B cells in the spleen and lymph nodes revealed the presence of the same kinetically distinct populations (Fig. 2) with rapid turnover of the $B220^{lo}/HSA^{hi}$ cells and slow renewal of the $B220^{hi}/HSA^{lo}$ cells. Comparison with F_1 mice was undertaken in view of preliminary work suggesting that they were more resistant to the toxic effects of prolonged BrdU administration than B6 mice (data not shown). In practice, uptake of BrdU at 7 d was similar in the two strains (Fig. 2) and no evidence of increased toxicity in B6 mice was observed at the relatively low dose of 0.25 mg/ml used. These observations remained valid during prolonged BrdU loading for up to 6 wk (data not shown). Consequently, transgenic mice on the

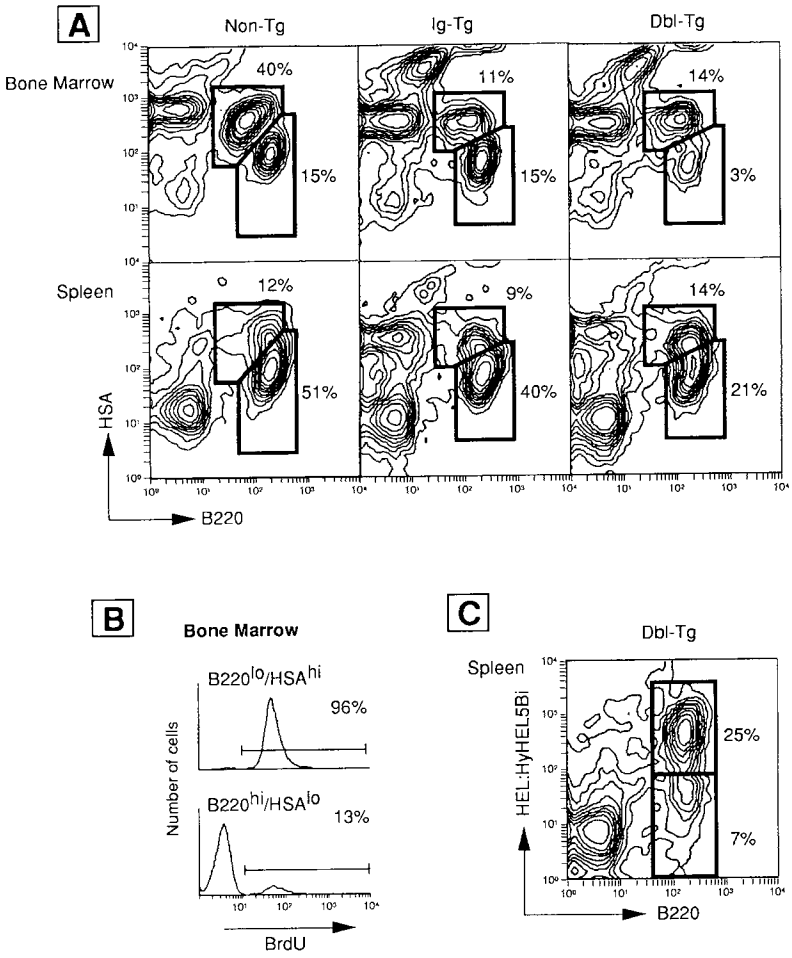

Figure 1. Analysis gating for BrdU uptake. (*A*) Bone marrow and spleen from non-Tg, Ig-Tg, and ML5 Dbl-Tg mice on a B6 background. Two distinct B220-positive populations were discernable in the bone marrow, namely $B220^{lo}/HSA^{hi}$ cells and $B220^{hi}/HSA^{lo}$ cells. Although the spleen shows a more continuous range of B220 and HSA expression, analysis gates defined by expression in the marrow were applied. The 10% probability contour plots were gated on small lymphocytes by forward and orthogonal light scatter. Percentages shown represent values from individual mice; the means (± SD) of values for $B220^{lo}/HSA^{hi}$ and $B220^{hi}/HSA^{lo}$, respectively, from the cohort of mice used in the experiment detailed in the text were as follows: Spleen, Non-Tg, 14 ± 2.2 and 47 ± 4.9%; Ig-Tg, 12 ± 3.4 and 36 ± 6.8%; Dbl-Tg, 13 ± 2.8 and 15 ± 4.9%; Bone marrow values shown in Table 1. (*B*) BrdU fluorescence histograms from the bone marrow of a non-Tg B6 mouse after 1-wk's loading with BrdU. Uptake was determined by gating on $B220^{lo}/HSA^{hi}$ cells (*top*) or $B220^{hi}/HSA^{lo}$ cells (*bottom*) being 96 and 13%, respectively, as shown in Fig. 2. (*C*) Spleen cells from a Dbl-Tg mouse showing the gates applied to determine the BrdU uptake of HEL-binding and non-HEL-binding B cells. 10% probability contour plot.

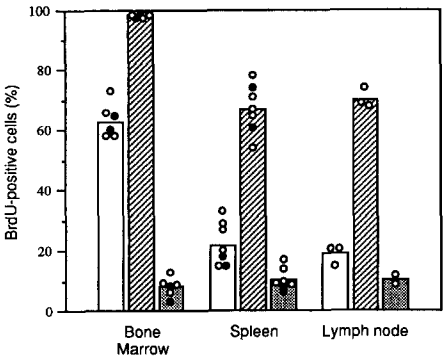

Figure 2. BrdU uptake in bone marrow, spleen, and lymph node of adult (age, >3 mo) B6 (*open circles*) or (B6 × SJL/J)F_1 mice (*closed circles*) given BrdU for 7 d. Gates shown in Fig. 1 *A* were applied to determine BrdU uptake in B220-positive cells (*unshaded bars*), immature $B220^{lo}/HSA^{hi}$ cells (*hatched bars*), or mature $B220^{hi}/HSA^{lo}$ (*stippled bars*). The relative proportions of the two populations varied in the three tissues examined; $B220^{lo}/HSA^{hi}$ cells represented 56% of marrow B220-positive cells, 25% of splenic B220-positive cells, and 12% of lymph node B220-positive cells.

two backgrounds were used interchangeably in subsequent experiments (see below).

The findings described here established that immature $B220^{lo}/HSA^{hi}$ and mature $B220^{hi}/HSA^{lo}$ B cells from both central and peripheral lymphoid tissue of non-Tg mice display distinct kinetic profiles. Three-color FACS® analysis using B220 and HSA was therefore repeated on B cells from MD4 Ig-Tg and ML5 Dbl-Tg mice on a B6 background. As shown in Fig. 1 *A*, the FACS® profiles for Ig-Tg and Dbl-Tg B cells were similar to those from non-Tg mice. Moreover, in the tolerant Dbl-Tg mice, by substituting HEL/HyHEL5Bi for HSA during the staining procedure, it was possible to subdivide B cells into two further populations on the basis of their capacity to bind HEL (Fig. 1 *C* and see below).

Higher Rate of BrdU Uptake in B Cells from Dbl-Tg Mice. Based on the labeling technique described in the previous section, the life span of anergic B cells was determined by comparing their turnover in ML5 Dbl-Tg mice with that in Ig-Tg mice on a B6 background during continuous loading with BrdU for 6 wk. At all timepoints analyzed, the BrdU uptake by B cells from Dbl-Tg mice was significantly higher than in Ig-Tg mice, resulting in a fivefold reduction in their 50% renewal time (Fig. 3 *A* and Table 1). When the rapidly labeling $B220^{lo}/HSA^{hi}$ cells were excluded to restrict analysis to mature $B220^{hi}/HSA^{lo}$ cells, the higher BrdU-uptake by Dbl-Tg B cells was still clearly evident, the 50% renewal time being decreased fourfold (Fig. 3 *B* and Table 1). The rate of BrdU uptake by Ig-Tg B cells was similar to that of non-Tg mice (Table 1) and was consistent with earlier life span estimates of 5–6 wk (19, 20).

BrdU uptake by B220-positive cells from both Ig-Tg and Dbl-Tg mice displayed a second-order kinetic pattern with an initial rapid labeling phase over the first 1–2 wk followed by a slower increase thereafter. In Ig-Tg mice, this effect disappeared when the newly generated $B220^{lo}/HSA^{hi}$ cells were excluded from the analysis (Fig. 3 *B*). By contrast, anergic Dbl-Tg B cells displayed a second-order pattern whether or not the $B220^{lo}/HSA^{hi}$ cells had been excluded (Fig. 3 *B*). This was subsequently shown to be due to a difference in kinetics of HEL-binding vs. non-HEL-binding B cells (see below).

A similar trend was observed when the experiments were repeated in Dbl-Tg and Ig-Tg mice bred on a (B6 × SJL/J)F_1 background. Thus, there was a markedly reduced 50% renewal time of B cells from the Dbl-Tg mice, the de-

Table 1. *Comparison of B Cell Composition and 50% Renewal Times in Non-Tg, Ig-Tg, and ML5 Dbl-Tg Mice*

	Spleen B cells		Bone marrow B cells		50% Renewal time	
Mouse strain	Total	$B220^{hi}/HSA^{lo}$	$B220^{lo}/HSA^{hi}$	$B220^{hi}/HSA^{lo}$	Total B cell	$B220^{hi}/HSA^{lo}$
	$\times 10^{-6}$		% of lymphocytes		wk	
B6 mice*						
Non-Tg	84 ± 21§	65 ± 16	29 ± 7.3	24 ± 9.5	3.9	5.1
Ig-Tg	28 ± 14	21 ± 9.8	12 ± 3.2	16 ± 8.2	3.4	5.0
Dbl-Tg	15 ± 6.4	8 ± 3.1	18 ± 3.1	4 ± 2.6	0.64	1.2
Reduction in Dbl-Tg	46%	62%	0%	75%	81%	76%
(B6 × SJL)F_1 mice‡						
Ig-Tg	55 ± 10	46 ± 7.8	15 ± 9.2	17 ± 3.0	5.4	7.0
Dbl-Tg	6.7 ± 3.4	3.1 ± 1.9	14 ± 4.8	1.0 ± 0.4	0.52	1.1
Reduction in Dbl-Tg	88%	93%	7%	94%	90%	84%

* Mice were 18–33 wk of age. Data were derived from 21 non-Tg, 13 Ig-Tg, and 10 Dbl-Tg mice.
‡ Mice were 23–40 wk of age. Data were derived from eight Ig-Tg and nine Dbl-Tg mice.
§ Values are mean ± SD.

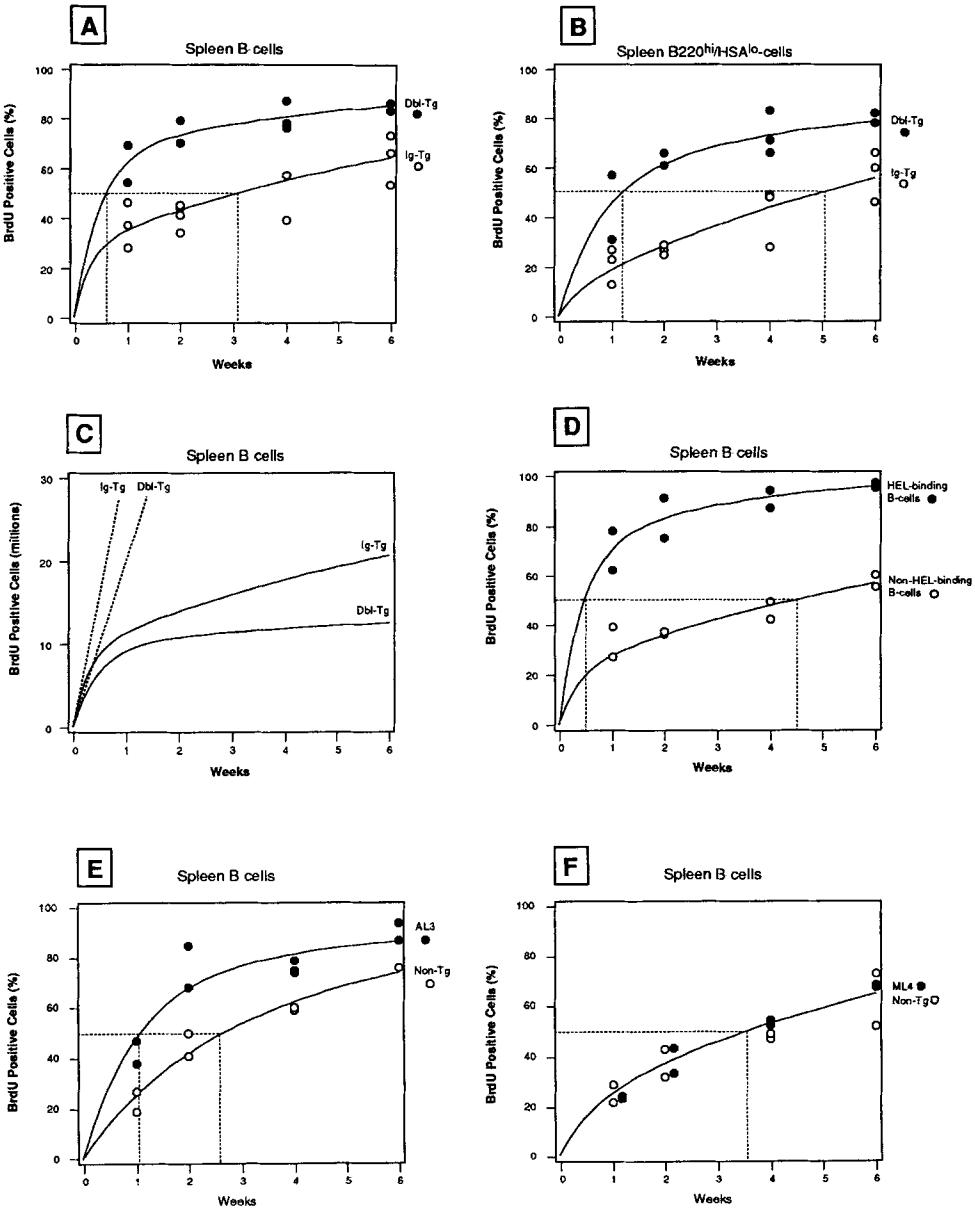

Figure 3. (*A–D*) BrdU uptake in splenic B cells from Ig-Tg and Dbl-Tg B6 mice during a 6-wk loading period. (*A*) Gating on all B220-positive cells. (*B*) Gating on $B220^{hi}/HSA^{lo}$ cells. (*C*) Percentage uptake curves from *A* corrected for absolute numbers of B cells. (*D*) Turnover of splenic B cells in Dbl-Tg mice based on their ability to bind HEL. The mean age of mice at commencement of BrdU loading was 24 wk (range, 18–30 wk). (*E* and *F*) BrdU uptake in splenic B cells from radiation chimeras produced by reconstituting non-Tg (*open circles*) or sHEL-Tg (*closed circles*) B6 mice with MD4 Ig-Tg bone marrow. (*E*) sHEL-Tg line AL3, which expresses high serum levels of HEL (20–110 ng/ml); (*F*) sHEL-Tg line ML4, which expresses low HEL levels (<1 ng/ml). Where necessary, plotted points have been staggered when overlapping with others. Values are from individual mice. Curves represent line of best fit.

crease affecting both total B cells and $B220^{hi}/HSA^{lo}$ cells (Table 1).

B Cell Numbers and Cell Cycle Analysis in Ig-Tg and Dbl-Tg Mice. The higher rate of BrdU uptake by anergic B cells from Dbl-Tg mice is consistent with a reduction in life span. In theory, however, this could have been due to an increase in their rate of cell division as a result of persistent exposure to antigen. Indeed, although the Dbl-Tg mice were tolerant in vivo, the anergic B cells had been shown to proliferate better than their Ig-Tg counterparts after removal from the tolerant environment and stimulation in vitro with LPS (21) or a T cell membrane preparation containing the CD40 ligand (Eris, J., A. Basten, M. Kehry, and P. Hodgkin, manuscript submitted for publication) even in the presence of HEL. To exclude this alternate explanation for the increase in BrdU incorporation by anergic B cells, the absolute numbers of B cells, the rate of splenic input of B cells from the bone marrow, and the proportion of B cells entering the cell cycle were determined in Dbl-Tg vs. Ig-Tg mice.

B cell populations from the bone marrow and spleen of the same Ig and Dbl-Tg mice used in the previous section were analyzed for cell numbers and maturity. The proportion of small bone marrow lymphocytes (by foward and side scatter) that were of immature $B220^{lo}/HSA^{hi}$ phenotype was similar in Ig-Tg and Dbl-Tg mice on both B6 and (B6 × SJL/J)F_1 backgrounds (Table 1). However, the proportion of mature $B220^{hi}/HSA^{lo}$ cells in the marrow was reduced 4-fold in Dbl-Tg compared with Ig-Tg mice on a B6 background, and more than 10-fold in those bred on the (B6 × SJL/J)F_1 background. These findings were mirrored in the spleen, where

a marked decrease in total B cells and B220^{hi}/HSA^{lo} B cells in Dbl-Tg compared with Ig-Tg mice was again observed, closely matching the reduction in 50% renewal time (Table 1). Although such a marked decrease in B cell numbers had not been reported previously in Dbl-Tg mice, the animals from earlier experiments were much younger (~6 wk of age) than those used in the present study (18–33 wk). BrdU loading experiments have been developed and are currently underway to determine precisely what changes in B cell number and life span do occur as the transgenic mice mature.

The rate of input of newly labeled B cells into the spleen was estimated by applying the BrdU uptake curves to the total B cell numbers in Ig-Tg and Dbl-Tg mice. The rates of input of total B cells and of mature B220^{hi}/HSA^{lo} cells (given by the slopes of the respective curves at the origin) were similar in the two groups (Fig. 3 *C*), making it unlikely that an alteration in generation or export of B cells from the marrow was the explanation for the difference in B cell numbers. Moreover, the finding speaks against the possibility of enhanced proliferation by B cells in the peripheral lymphoid tissue of Dbl-Tg mice.

Finally, the background rate of proliferation of splenic B cells from Ig compared with Dbl-Tg mice was measured by analyzing their DNA content on the basis of B220 expression or their ability to bind HEL. Normal age-matched B6 or (B6 × SJL/J)F_1 mice served as controls. The proportion of cycling B cells was lower in both the Ig-Tg and Dbl-Tg mice than in controls (Fig. 4), perhaps reflecting a restriction in antigen–receptor repertoire. On the other hand, no significant differences in the percentages of cycling cells were observed between Ig-Tg and Dbl-Tg mice, whether they were analyzed by B220 expression or by HEL-binding. Taken together, these findings support the conclusion that anergic B cells in Dbl-Tg mice do indeed have a markedly reduced life span in vivo.

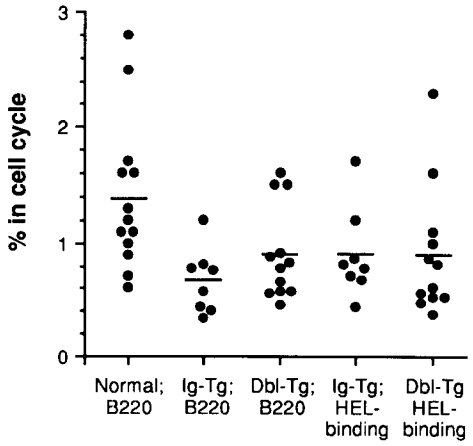

Figure 4. Cell cycle analysis of B220-positive, or HEL-binding, splenic cells from Non-Tg (B6 or (B6 × SJL/J)F_1 strain), Ig-Tg or ML5 Dbl-Tg (B6 strain) mice aged between 7 wk and 9 mo. Percentages of cells out of $G_{0/1}$ phase are shown, with horizontal bars representing the means. Positive controls consisting of bone marrow B cells showed rates of between 7 and 15% (data not shown).

Reduced Life Span of Anergic B Cells Is Due to Rapid Turnover of HEL-Binding Cells. Tolerance induction in B cells from Dbl-Tg mice depends on a threshold occupancy of Ig-receptors by HEL of between 5 and 45% (22). The reduction in life span of anergic B cells was presumably a result of antigen binding to their surface receptors. To confirm this, use was made of the fact that a small proportion of B cells from Dbl-Tg mice fails to bind HEL despite continued expression of the transgenic IgH^a marker, due to endogenous light chain rearrangement (Crosbie, J., unpublished observations). Hence it was possible to separate the B220-positive cells of Dbl-Tg mice into HEL-binding and non-HEL-binding populations (Fig. 1 *C*) and to determine the life span of each population separately by loading with BrdU over the standard 6-wk period.

Approximately 75% of splenic B cells bound HEL while the remainder did not. The HEL-binding B cells were very short-lived, with a 50% renewal rate of 3.4 d, whereas the non-HEL-binding B cells had a 50% turnover almost 10 times as great, namely 4.6 wk (Fig. 3 *D*), which was similar to the rate of renewal of Ig-Tg B cells (Fig. 3 *A* and Table 2). These results therefore provided a powerful internal control for the studies performed in individual Ig- or Dbl-Tg mice and reconfirmed the finding of a markedly reduced life span for anergic B cells.

Life Span of Ig-Tg B Cells Is Reduced in sHEL-expressing Chimeras. The differential renewal time of B cells from Ig vs. Dbl-Tg mice, and of HEL-binding vs. non-HEL-binding B cells in Dbl-Tg mice, confirmed that the reduced life span of anergic B cells is a consequence of antigen binding to the surface receptors. However, antigen expression in the ML5-strain mice is under control of the metallothionein promoter, which may lead to synthesis of HEL in addition to small amounts of anti-HEL antibody by Dbl-Tg B cells (23). Consequently the reduction in life span of anergic B cells might have been artifactual due to complexing of antigen and antibody within or on the surface of these cells. This possibility was obviated by reconstituting irradiated ML5 sHEL-Tg mice with bone marrow from MD4 Ig-Tg donors, with non-Tg

Table 2. *Comparison of B Cell Composition and 50% Renewal Times in Ig-Tg and ML5 Dbl-Tg Mice, Gating on HEL or non-HEL-binding B Cells*

Mouse strain	Spleen B cells	50% Renewal time
	10^{-6}	*wk*
Ig-Tg*		
(HEL binding)	28 ± 14	3.4
Dbl-Tg		
(Non-HEL binding‡)	3.4 ± 1.2	4.6
Dbl-Tg		
(HEL binding‡)	10.5 ± 3.4	0.49

* From Table 1.
‡ B6 strain mice at 18–33 wk of age; data were derived from eight mice.

recipients serving as controls. Both groups of mice were then loaded with BrdU over 6 wk as previously described in Materials and Methods.

A significant reduction in B cell life span in sHEL-Tg recipients compared with non-Tg recipients was observed, as demonstrated by a more rapid accumulation of BrdU-positive cells and a decline of similar magnitude in total and mature $B220^{hi}/HSA^{lo}$ B cell numbers in the former mice (Table 3). Although the decrease in 50% renewal time was less dramatic than in intact Dbl-Tg mice, the difference between sHEL-Tg recipients and controls was clear cut and confirmed that the reduced life span of anergic B cells is a real phenomenon.

Reduction in Life Span of Dbl-Tg B Cells Correlates with the Anergic State. Clearly B cells rendered anergic by encounter with soluble self-antigen have a significantly reduced life span. The question therefore arose as to whether the decrease in life span is a property of the anergic B cells per se or reflects the concentration of sHEL and the degree of receptor occupancy by antigen on the B cell membrane. To distinguish between these two possibilities BrdU uptake was measured in chimeras constructed by transferring MD4 Ig-Tg bone marrow cells into sHEL-Tg recipients from two other lines (AL3 and ML4) expressing different concentrations of antigen under control of the albumin and metallothionein promoters, respectively. The AL3 line was selected since the mean serum level of HEL (20–110 ng/ml) is up to four to five times higher than that of ML5 (15–20 ng/ml), and the B cell compartment is, as expected, tolerant (our unpublished observations). By contrast, ML4 mice express very low levels of sHEL (<1 ng/ml), which induces tolerance in the T cell but not the B cell compartment and therefore is not associated with B cell anergy in ML4 Dbl-Tg mice (11).

When the kinetic activity of B cells from AL3 chimeras was compared with that in non-Tg controls, a marked decrease in 50% renewal time was again observed, but both the degree of the reduction and the associated fall in B cell numbers was almost identical to the results obtained for B cells from ML5 recipients (Fig. 3 *E* and Table 3). By contrast, in the ML4 recipients, no significant reduction in B cell numbers occurred (Table 3) and BrdU uptake was almost identical to that in non-Tg recipients (Fig. 3 *F* and Table 3). When taken in the context of the previous findings, the data provide strong support for the concept that the reduction in life span of B cells from tolerant mice is related to the anergic state per se and antigen concentration in serum or on surface Ig receptors is only important in attaining the critical threshold necessary for induction of B cell anergy.

Table 3. *Comparison of B Cell Composition and 50% Renewal Times in Non-Tg or sHEL-Tg Radiation Chimaeras, Reconstituted with MD4 Ig-Tg Bone Marrow*

	Spleen B cells		Bone marrow B cells		50% Renewal time	
Transgene combination	Total	$B220^{hi}/HSA^{lo}$	$B220^{lo}/HSA^{hi}$	$B220^{hi}/HSA^{lo}$	Total B cell	$B220^{hi}/HSA^{lo}$
	$\times 10^{-6}$		*% of lymphocytes*		*wk*	
ML5 Recipient chimeras*						
Non-Tg	61 ± 22	52 ± 20	13 ± 4.4	31 ± 9.2	3.8	4.9
sHEL-Tg	19 ± 12	10 ± 7.7	14 ± 5.6	2.5 ± 1.6	1.1	2.0
Reduction in sHEL-Tg	69%	81%	0%	92%	71%	59%
AL3 Recipient chimeras‡						
Non-Tg	71 ± 17	55 ± 14	19 ± 7.1	24 ± 6.4	2.7	3.8
sHEL-Tg	22 ± 7.4	14 ± 5.1	21 ± 6.6	4.7 ± 2.4	1.1	2.0
Reduction in sHEL-Tg	69%	75%	0%	80%	59%	47%
ML4 Recipient chimeras§						
Non-Tg	41 ± 8.1	31 ± 6.1	–	–	3.9	5.1
sHEL-Tg	46 ± 18	32 ± 13	–	–	3.4	4.8
Reduction in sHEL-Tg	0%	0%	–	–	13%	6%

ML5, AL3, and ML4 are sHEL-Tg lines that express moderate (15–20 ng/ml), high (20–110 ng/ml), or low serum HEL levels, respectively.
* ML5 mice were on a (B6 × SJL/J)F_1 background and were used 9 wk after bone marrow reconstitution. Data were derived from 10 non-Tg and 10 ML5 sHEL-Tg recipients.
‡ AL3 mice were on a B6 background and were used 9 wk after bone marrow reconstitution. Data were derived from seven non-Tg and nine AL3 sHEL-Tg recipients.
§ ML4 mice were on a B6 background and were used 19 wk after bone marrow reconstitution. Data were derived from eight non-Tg and eight ML4 sHEL-Tg recipients.

Discussion

Self-tolerance in the B cell repertoire is mediated predominantly by either deletion or anergy (6, 24–26). Since selection is thought to be involved in both these processes, measurement of life span of tolerant B cells with a DNA precursor like BrdU has the potential to shed additional light on the cellular mechanisms underlying each of them. To obtain meaningful results on the life span of self-reactive B cells with a single antigenic specificity, use was made of our well-defined double transgenic model of self-tolerance in which monoclonal populations of HEL reactive B cells exposed to soluble or membrane-bound HEL are rendered anergic (9, 22) or undergo deletion (7), respectively.

Initially it was important to establish that the BrdU labeling technique could distinguish clearly between immature and mature B cells in the bone marrow and peripheral lymphoid tissue since induction of tolerance to soluble HEL has the potential to influence the life span of B cells at any stage during maturation. This goal was achieved by performing three-color FACS® analysis on lymphoid cells from nontransgenic mice with monoclonal antibodies to the reciprocally expressed B cell maturation markers B220 and HSA as well as BrdU (Fig. 1). In the bone marrow of nontransgenic mice given BrdU for 1 wk, almost 100% of immature $B220^{lo}/HSA^{hi}$ B cells were labeled and remained so for as long as BrdU was given (Fig. 2 and data not shown) (18). Thus, the entire cohort of B cells exported from the bone marrow to the periphery were BrdU positive, which meant that the rate of appearance of labeled cells in the spleen or lymph node represented the turnover of that population and hence its average life span, as reported previously (19). B cells with the $B220^{lo}/HSA^{hi}$ phenotype in the periphery, like those in the bone marrow, were rapidly labeled whereas uptake of BrdU by mature $B220^{hi}/HSA^{lo}$ cells occurred more slowly. Hence the short- and long-lived B cell populations corresponded closely to the immature and mature B cell subsets defined by reciprocal expression of B220 and HSA. In addition, the same clearcut distinction in life span between the two populations of B cells was observed in Ig and Dbl-Tg mice when cells from their lymphoid tissues were subjected to similar FACS® analysis (Figs. 1 *A* and 3, and data not shown).

By comparison with mature Ig-Tg B cells, anergic $B220^{hi}/HSA^{lo}$ B-cells from tolerant ML5 Dbl-Tg mice incorporated BrdU significantly more rapidly (Fig. 3). The decreased life span was not due to an intracellular interaction between antibody and antigen within the cells, since a comparable difference in BrdU uptake occurred in radiation chimeras produced by reconstituting ML5 sHEL-Tg mice with MD4 Ig-Tg bone marrow (Table 3 and Fig. 3, *E* and *F*). Moreover the proportion of mature B cells in cycle (Fig. 4) and the rate of entry of newly generated B cells into the spleen of Dbl-Tg mice (Fig. 3 *C*) were comparable to those in the Ig-Tg controls despite being exposed to sHEL throughout their development. These data, when taken in conjunction with the reduction in total and $B220^{hi}/HSA^{lo}$ B cell numbers (Tables 1 and 3), indicate that anergic B cells do indeed have a reduced life span.

The differential rates of B cell turnover were even more dramatic when analysis was restricted to HEL-binding cells alone; in this case HEL-binding anergic B cells died within 3–4 d of reaching the spleen, while non-HEL-binding B cells had a renewal rate of 4–5 wk similar to B cells from non-tolerant Ig-Tg mice. Consequently it was predictable that the shortened life span of mature B cells from Dbl-Tg mice proved to be associated with their anergic state (Fig. 3, *E* and *F*, and Table 3). In other words, receptor occupancy by HEL above the critical threshold required to induce tolerance appeared to be responsible for the observed reduction in life span presumably due to downstream effects on intracellular signaling pathways. Consistent with this conclusion was the normal life span of B cells from ML4 chimeras that were not tolerant but rather displayed clonal "ignorance" of HEL (24).

In previous studies Ig-Tg B cells exposed to HEL in multivalent membrane-bound rather than soluble form during early development in the bone marrow were shown to undergo maturation arrest followed by rapid deletion within ~15 h, which prevented their release into the periphery (8). Although exposure of Ig-Tg B cells to sHEL in the bone marrow did not prevent migration to the spleen, once there, those B cells capable of binding HEL were apparently eliminated within 3–4 d. This 10-fold reduction in life span, although less efficient than that observed in the presence of membrane HEL, is consistent with significant purging of self-reactive B cells.

The mechanism responsible for the shortened life span of mature self-reactive B cells is unclear. According to conventional dogma, deletion depends on cross-linking of Ig-receptors above a critical threshold (27). In the current model, this may have occurred in sHEL Dbl-Tg mice as a result of encounter with high concentrations of cross-linked HEL on the follicular dendritic cells in the spleen or by interaction with circulating multivalent antigen since lysozymes including HEL are known to complex with serum proteins such as α-2 macroglobulin (reference 28 and Fulcher, D., and P. Peake, unpublished observations) or with themselves. Alternatively the reduced life span of anergic B cells could have been due to negative signaling related to binding of self-antigen at an immature stage of development in the bone marrow (27) and/or by the failure to receive T cell help. To distinguish between these two possibilities it would be interesting to determine the fate of immature vs. mature Ig-Tg B cells after transfer into soluble HEL-Tg recipients. Irrespective of the mechanism, however, the data on life span presented here suggest that anergy and deletion may not be entirely discrete entities, but rather represent a continuum with the anergic state being the harbinger of delayed deletion.

If anergy is indeed a form of delayed deletion and anergic self-reactive B cells have a relatively short life span in peripheral lymphoid tissue, these findings have significant implications for our understanding of self-tolerance and autoimmunity. For example the opportunity for B cells with specificity for soluble self-antigen to escape from self-tolerance and to mount a pathogenic autoimmune response is reduced since

deletion is the most efficient way of maintaining the tolerant state. Interestingly, it is now apparent from work in our model and those of others (Crosbie, J., unpublished observations, and references 30–32) that Ig-Tg B cells can lose self-reactivity and escape deletion by the process of receptor editing. The current experiments are consistent with such a conclusion since a loss of HEL binding was associated with a return in B cell life span to control values (Fig. 3 *D*). It remains to be determined whether this involves active rearrangement of endogenous light chain genes by expression of recombinase activator genes (31), or whether it involves the selection of rare cells that have suppressed their light chain transgene during development in favor of the endogenous gene in a stochastic manner. In either case, an attempt by anergic B cells to rearrange endogenous light chain genes in order to escape self-reactivity would allow them to generate potentially useful foreign specificities. On the other hand, anergic self-reactive B cells that are destined to die within a few days of reaching peripheral lymphoid tissue are unlikely to be particularly efficient in inducing unresponsiveness in the T cell repertoire as has recently been suggested by some investigators for small resting B cells (33, 34). Nevertheless, the inclusion of anergy within the spectrum of deletion does raise the question of whether the high attrition rate of newly generated B cells on reaching the periphery of normal mice, estimated to be ~50% (Fulcher, D., and A. Basten, manuscript in preparation), might be explained at least in part by an elimination mechanism of this kind with the potential to act at multiple stages during development of self-reactive B cells.

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Address correspondence to Dr. Antony Basten, Centenary Institute of Cancer Medicine and Cell Biology, University of Sydney, Newtown, NSW 2006, Australia.

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