Immature Surface Ig⁺ B Cells Can Continue to Rearrange \( \kappa \) and \( \lambda \) L Chain Gene Loci

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Summary

Pro and pre B cells possess the long-term capacity to proliferate in vitro on stromal cells and interleukin 7 (IL-7) and can differentiate to surface immunoglobulin (slg⁺) cells upon removal of IL-7 from the cultures. A key event in this differentiation is the extensive cell loss due to apoptosis. Because the proto-oncogene \( bcl-2 \) can promote cell survival, we established pre-B cell lines from \( E\mu-bcl-2 \) transgenic mice. These pre-B cells have the same properties as those derived from non-\( bcl-2 \) transgenic mice except that they do not die by apoptosis. This allowed us to study the fate of newly formed B cells in vitro for a longer period of time. Here we show that early during the differentiation of pre-B cells, upregulation of RAG-1 and RAG-2 expression go hand in hand with rearrangements of the Ig gene loci. Moreover, the newly formed slg⁺ B cells continue to express RAG-1 and RAG-2 and continue to rearrange \( \lambda \) chain gene loci, even in the absence of proliferation, in an orderly fashion, so that \( \kappa \)L⁺ slg⁺ cells can become \( \lambda \)L⁺ slg⁺ or slg⁻ cells, whereas \( \lambda \)L⁺ slg⁻ cells can become slg⁻, but not \( \kappa \)L⁺ slg⁻ cells. Thus, deposition of a complete Ig molecule on the surface of a B cell does not automatically stop the Ig-rearrangement machinery.

The \( bcl-2 \) gene encodes a 26-kD \( \alpha \) protein and a \( \beta \) form of 21 kD varying at the COOH-terminal end (1). The \( bcl-2 \) protein is mainly expressed in B- and T-lymphocyte-lineage cells at defined stages of their differentiation (2–4). It was originally identified as the locus translocated to the Ig H chain locus in the (t 14:18) (q32;q21) translocations found in most cases of human follicular lymphomas (5–9). Translocations leave the coding regions intact but put the (onco)gene under the control of expression of the IgH locus, leading to constitutive high expression along the B-lineage pathway from early precursors to plasma cells. Although the exact functions of the \( bcl-2 \) gene are still unknown, it plays the role of rescuing cells from programmed cell death (apoptosis, characterized by chromatin condensation and by endonucleolytic cleavage of DNA into characteristic nucleosome ladders) (10, 11), thereby prolonging the lifespan of the cell in which it is expressed. This has been shown for B-lineage, T-lineage, and other hematopoietic cells by transfection of the \( bcl-2 \) gene (12, 13) and in \( bcl-2 \)-transgenic (tg) mice (14–20). The \( bcl-2 \) expression pattern in normal pre-B and -related genes such as \( V_{\mu} \) and \( \lambda \)s (24), and RAG-1 and RAG-2 (25, 26), and their use of \( \kappa \)L and \( \lambda \)L chain gene loci during differentiation to slg⁺ B cells.

Materials and Methods

Animals and Cell Lines. \( E\mu-bcl-2 \) transgenic mice originally obtained from Drs. A. Harris and S. Cory, (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and previously described (16–18) were bred in the breeding facilities of the
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The presence of the bcl-2 tg was ascertained by the presence of SV-40 DNA sequences flanking the bcl-2 tg (16–18). The stromal cell line PA-6 was obtained from Dr. H. A. Kodama (Osaka University, Japan). PA-6 cells and 3T3 fibroblasts transfected with the murine IL-7 cDNA were grown as described (23).

Interleukins, Monoclonal Antibodies, Immunofluorescence, and Flow Cytometric Analysis. rIL-7, produced by the fibroblast cell line 3T3 transfected with the mouse IL-7 gene, was used at a concentration of 50–200 U/ml. The FITC-, PE- or biotin-conjugated mAb RAB-7 (anti-CD43), Ack4 (anti-c-kit), M41 (anti-αH chain), 187.1 (anti-κL chain), and 4/1–101 (anti-λL chain) were purified from culture supernatant and conjugated as previously described (23). Cell surface immunofluorescence and flow cytometric analyses were performed as described (27). Cells expressing κ or λ L chains were sorted using the FACStar Plus® (Becton and Dickinson, Mountain View, CA).

Preparation of Pre-B Cell Lines. Pre-B cell lines that possess the long-term capacity to proliferate on stromal cells in the presence of IL-7 were prepared from day 17–18 fetal liver and from 2-wk-old bone marrow cells as previously described (23, 27). Throughout these experiments PA-6 cells were used as stromal cells. The influence of hydroxyurea on the proliferation and differentiation of our pre-B cells was studied using different concentrations. For proliferation, pre-B cell cultures were initiated at 5 × 10⁴ cells/ml on PA-6 plus IL-7 in the presence of different concentrations of hydroxyurea. Cell growth was determined by [H]thymidine uptake during the last 6 h of a 72-h culture period. For differentiation, pre-B cell cultures were initiated at 1–2 × 10⁵ cells/ml on PA-6 stromal cells without IL-7 and in the presence of different concentrations of hydroxyurea. Differentiation was determined by measuring Ig* cells after a 72-h culture period.

Northern Blot and Dot Blot Analysis. Total cellular RNA was extracted from cells with acid guanidinium-thiocyanate-phenol-chloroform (28). For Northern blot analysis 15 μg denatured total RNA per lane was fractionated on a 1.2% agarose-0.4 M formaldehyde gel and then blotted onto Gene Screen Plus® nylon membranes (Du Pont-Biotechnology Systems NEN, Boston, MA) by downward alkaline blotting. For dot blot analysis, 15 μg total RNA was denatured in 55% formamide, 7% formaldehyde, 50 mM Tris-C1, pH 8.3, leading to DNA equivalents of 300, 100, 30, 10, and 3 cells, respectively, per 5 μl of the dilution. The PCR reactions were performed according to the enzyme manufacturer’s conditions (Boehringer Mannheim). The temperature settings for the Perkin Elmer Cetus PCR Cycler (Norwalk, CT) were 94°C 20 s, 58°C 20 s, 72°C 30 s, and 72°C 2 min. PCR reactions were performed on 1 μl of the cDNA preparation (i.e., RNA from 1,000 cells) in a total volume of 20 μl according to the enzyme manufacturer’s conditions (Boehringer Mannheim). The temperature settings for the Perkin Elmer Cetus PCR Cycler (Norwalk, CT) were 94°C 20 s, 58°C 20 s, 72°C 40 s for 35 cycles.

Semiquantitative PCR Titration for k Rearrangements. BCL2-5 cells on days 0, 1, 2, 3, and 4 of differentiation were counted by Trypan blue exclusion, lysed by boiling, and DNA prepared as described elsewhere (27). DNA was diluted in threefold steps in 10 mM Tris-Cl, pH 8.3, leading to DNA equivalents of 300, 100, 30, 10, and 3 cells, respectively, per 5 μl of the dilution. The PCR reaction was performed according to the enzyme manufacturer’s conditions (Boehringer Mannheim) in a final volume of 20 μl. The PCR primers (universal V, and J, primers detecting V,J, and V,J, rearrangements) and reaction conditions are described elsewhere (30). Half of the resulting PCR product was separated on a 1.5% agarose gel by electrophoresis. Bands of 536 (V,J,) and 130 bp (V,J,2) were visualized under UV light.

Results

Establishment of Pre-B Cell Lines from bcl-2 tg Mice. Pre-B-I cells of normal mice can proliferate for long periods of time on stromal cells in the presence of IL-7 (23, for the nomenclature of pre-B cells used by us see reference 24). To establish pre-B-I cell lines from bcl-2 tg mice, cell suspensions of fetal liver at day 17–18 of gestation and of bone marrow 2 wk after birth were cultured under conditions of limiting dilution on PA6 stromal cells in the presence of exogenously added IL-7. Growth of cells was scored at day 6 of culture, and lines and clones were established from positive cultures.
by continued growth on stromal cells in the presence of IL-7. Fetal liver cells were obtained from individual embryos of crosses of mice heterozygous for the \( bcl-2 \) \( tg \) and from bone marrow of \( bcl-2 \) \( tg \) mice. The presence of the \( bcl-2 \) \( tg \) in pre-B cell lines was ascertained by the presence of SV-40 DNA sequences flanking the \( bcl-2 \) gene construct, and was furthermore evident from the unusual survival capacity of cells in tissue culture upon differentiation as described below. Three \( bcl-2 \) transgenic cell lines (\( bcl-2-1 \), \( bcl-2-5 \), and \( bcl-2-6 \)) were derived from fetal liver of three individual embryos which were typed as \( tg \) positive.

Proliferation of \( bcl-2 \) \( tg \) pre-B cells on stromal cells in the presence of IL-7 was indistinguishable from pre-B cells of normal mice in several parameters. Both needed the simultaneous presence of stromal cells and IL-7 for proliferation, both proliferated at the same rates and for the same long periods of time (data not shown). The frequencies of pre-B cells (1 in 50 at day 17–18 of gestation in fetal liver; 1 in 50–100 in 2-wk-old bone marrow) were within the ranges found for normal cells.

All \( bcl-2 \) \( tg \) pre-B-I cell lines, just as those from normal mice, expressed mRNA for the pre-B cell-specific genes \( \nu \), \( RAG-1 \), and \( RAG-2 \), but not \( \kappa \) L and \( \lambda \) L chain genes, as detected in Northern blot analyses (Fig. 1). They expressed CD43, c-kit, B220, and BP-1, but not slg (\( \mu \), \( \kappa \)), as detected by immunofluorescence with specific mAbs. Pre-B-I cells from \( bcl-2 \) \( tg \) mice were found to be \( D_{2H}^{\text{m-r}} \)-rearranged in their H chain loci (data not shown).

We conclude that \( bcl-2 \) \( tg \) pro and pre-B-I cells have many of the properties of normal pre-B-I cells.

**Differentiation of \( bcl-2 \) \( tg \) Pre-B-I Cell Lines In Vitro**

Differentiation of normal pre-B-I cells occurs in vitro when IL-7 is removed from tissue culture (23). Differentiating cells lose the capacity to grow on stromal cells in the presence of IL-7 (23), and a portion of them become slg\(^+\) and mitogen-reactive cells within 2–3 d (23, 31, 32). Normal cells die rapidly by apoptosis, as evidenced by the endonucleolytic degradation of DNA and a loss of live cells (23).

When IL-7 was removed from cultures of \( bcl-2 \) \( tg \) pre-B-I cell lines, the cells lost the capacity to grow on stromal cells in the presence of IL-7, determined as frequencies of clonable cells, at a similar rate as pre-B-I cells of normal littermates (Fig. 2, bottom). In contrast to normal pre-B-I cells, \( bcl-2 \) \( tg \) pre-B cells did not die by apoptosis (Fig. 2, top). Cells remained viable during differentiation. However, the vast majority of the cells changed their phenotype. Within 3 d

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**Figure 1.** Northern blot (right) and RNA dot blot (left) analysis of undifferentiated and differentiating pre-B cell lines from \( E_{\mu}\)-\( bcl-2 \)-transgenic (\( bcl-2-5-4 \), \( bcl-2-5 \)) and normal (\( C57BL/6 \times DBA/2 \))\( F_1 \) mice (PAL.1). Pre-B cells were cultured and induced to differentiate as described (23). RNA dot blots with serial threefold dilutions and Northern blots were performed with 15 \( \mu \)g total RNA. Blots were probed with \( ^{32}P \)-labeled, subcloned 400-600-bp fragments of the genes \( RAG-1 \), \( RAG-2 \), \( \nu \), \( C_C \), \( C_H \), and \( \beta \)-actin as indicated in the figure. The size of the bands detected by these probes is given in kb. Quantification of the signals was performed on a Phosphor Imager (Molecular Dynamics) with the Image Quant software package supplied by the manufacturer. The expression level of \( RAG-1 \), normalized to the signal for \( \beta \)-actin, increases 26-, 2.5-, and 18-fold for \( bcl-2-5-4 \), \( bcl-2-5 \), PAL.1, respectively, within 2 d of differentiation. \( RAG-2 \) expression is upregulated 21-fold for \( bcl-2-5-4 \) and 2.0-fold for \( bcl-2-5 \) within 3 d of differentiation and 7.3-fold for PAL.1 after 2 d of differentiation.
Days in culture without stromal cells and IL-7

Figure 2. Survival (top) and cloning frequencies on PA6 + IL7 (bottom) of El~-bcl-2 fetal liver-derived pre-B cell lines bcl-2-1 (●), bcl-2-5 (●), and bcl-2-6 (●) and the C57BL/6 x DBA/2 F1 fetal liver-derived pre-B cell lines, PAL1 (●), 18L2 (△), and PAB8 (●) after culture for 0-7 d on PA6 stromal cells without IL-7. Viable cells were determined by Trypan blue exclusion. Cloning frequencies were determined by limiting dilution analysis with live cells as described (27). Positive pre-B cell growth was determined by the use of an inverted microscope at 6 d after onset of the cultures. Similar results were obtained with BM-derived pre-B cell lines.

they lost surface expression of c-kit, CD43, BP-1, and the surrogate L chain Vρ/δ5. Fetal liver-derived cell lines were indistinguishable in these differentiation capacities from bone marrow-derived cells (data not shown).

On the level of specific RNA expression, the surrogate L chain disappeared within 2-3 d, whereas expression of κL chain mRNA became detectable after 2 d, and of λL chain mRNA after 3 d of differentiation (Fig. 1). Surprisingly, the expression of RAG-1 and RAG-2 mRNA actually increased for the first 2 d of in vitro differentiation, before it began to decrease. This increase was seen in El~-bcl-2-tg as well as in normal cells. Using a limiting dilution PCR, IgL chain rearrangements were analyzed. About 1 in 300 cells at day 0 had a rearrangement, this was 1 in 100 at day 1, and more than 1 in 10 from day 2 of differentiation onwards.

**Differentiation to slg⁺ B Cells Expressing Either κ or λL Chains.** Within 3 d of in vitro differentiation of normal as well as bcl-2 pre-B cells, ~10% of all cells became slg⁺. At the same time, >85% of the slg⁺ cells expressed κL chains (Fig. 3). Extensive apoptosis of the normal cells prevents analysis of cells which have differentiated in vitro for periods exceeding 3 d. Such an analysis could, however, be done with bcl-2-tg cells. It is evident from the data shown in Fig. 3 that the total number of slg⁺ B cells increased with time of differentiation in vitro, reaching levels of 40% slg⁺ cells. Furthermore, the ratio of κL chains versus λL chains expressed in these slg⁺ cells changed. At 3 d of in vitro differentiation it was near 5, and at one week or later around 0.7.

These results suggested that the differentiated cells might be capable of continuous, successive rearrangements in their κ and λL chain gene loci. This was substantiated by the fact that sorted κL⁺ slg⁺ and λL⁺ slg⁺ were still positive for RAG-1 and RAG-2 expression as determined by RT-PCR. Therefore, bcl-2-tg cells differentiated for 5 d in vitro were assayed again for κL⁺ slg⁺ and λL⁺ slg⁺ cells. Thereafter, they were recultured in the absence of IL-7 for a further 3 d and then assayed again for κL⁺ slg⁺, λL⁺ slg⁺, and slg⁻ cells. Of the κL⁺ slg⁻ cells, about half remained κL⁺ slg⁻ cells, 10% became λL⁺ slg⁻ cells, and ~40% changed to slg⁻ cells. Of the λL⁺ slg⁻ cells, 75-80% remained λL⁺ slg⁻ cells, ~20-25% became slg⁻ cells, whereas 1% or less became κL⁺ slg⁻ cells. Several experiments with different fetal liver- and bone marrow-derived El~-bcl-2-tg pre-B cell lines yielded similar results. Taken together, these results indicate that the slg⁺ cells differentiated in vitro from bcl-2-tg, and probably also from normal pre-B cells, can continue to rearrange L chain gene loci.

**Differentiation in the Presence of Hydroxyurea.** Hydroxyurea (HU) inhibits the replication of DNA and, thereby, the proliferation of cells. It was added to El~-bcl-2-tg pre-B cells cultured on stromal cells in the presence or absence of IL-7 to see whether it would inhibit pre-B cell proliferation, and
whether that would still allow differentiation to slg+ B cells. Results in Fig. 4 show that HU at 10^-4 M or higher completely inhibits proliferation as measured by uptake of radioactive thymidine. On the other hand, the proportion of slg+ B cells differentiating at these high concentrations of HU remained constant. The development of Kl+ slg+ and λL+ slg+ B cells within 3 d in the presence of 2 × 10^-4 M HU yielded ratios of λL to K chain-expressing slg+ B cells indistinguishable from those obtained in the absence of HU (Fig. 3). The results collectively suggest that differentiation to slg+ B cells and maybe even the continued rearrangement of L chain gene loci can occur in the absence of DNA synthesis and cell proliferation.

Discussion

Pre-B cells that possess the long-term capacity to proliferate on stromal cells and IL-7 can differentiate to slg+ B cells by removal of IL-7 from the culture system (23, 31, 32). So far, extensive apoptosis (23) has hampered a more detailed analysis at the cellular and molecular level during this differentiation. Here we show that constitutive expression of the bcl-2 proto-oncogene in these pre-B cells can prevent this apoptosis and therefore allows us to follow the fate of newly formed slg+ B cells in vitro. One important question concerning the relevance of these studies is whether constitutive expression of the bcl-2 gene interferes with the normal pre-B cell differentiation pathway. The findings that the pre-B cells derived from Eμ-bcl-2 transgenic mice show the properties as those derived from non-bcl-2 transgenic mice like: (a) loss of clonability on stromal cells and IL-7 after removal of IL-7 from the culture system; (b) loss of markers like CD43, c-kit, BP-1, VpreB, and λ5 during differentiation; (c) upregulation of RAG-1 and RAG-2 expression during differentiation; and (d) identical kinetics for the appearance of slg+ B cells strongly argue that constitutive expression of bcl-2 does not interfere with the normal pre-B cell differentiation pathway. The κ/λ ratio of Ig molecules in the serum as well as that of membrane-bound Ig on B lymphocytes in mice is >10. These high ratios of κ to λ can be explained by a regulatory model, in which rearrangements at the λ loci are postulated to commence only after the κ loci have been exhaustively and nonproductively rearranged. This model is supported by the fact that most of the κ chain-expressing B cells have their λ chain genes in germline configuration, whereas in λ chain-producing B cells, the κ chain genes are frequently rearranged. (35-36).

High κ/λ ratios can also be explained by stochastic models that postulate an intrinsic rearrangement activity which is higher for κ than for λ loci (36-39). The stochastic model has recently gained support from studies with mice in which the Ig κ locus was inactivated (40-42). These studies demonstrate that rearrangements at the Ig λ locus and the production of XL chain-expressing B cells can occur without rearrangements at the Ig κ locus.

A third model argues that high κ/λ ratios in the periphery reflect antigen selection and stimulation and that newly generated B cells have κ/λ ratios near unity (42). Our earlier published findings (31) as well as the data shown above have strongly argued against this model. We have shown that immature slg+ B cells obtained from normal non-bcl-2 tg pre-B cells that differentiate in vitro in the absence of exogenous antigen have already κ/λ ratios of >10.

Our findings described here support the scenario in which κ gene rearrangements are initially preferred over λ genes. However, when the immature B cells can survive longer, the chances for λ chain gene rearrangements increase and consequently the ratio of κ over λ light chain-expressing B cells decreases. These and other findings (35-41) reaffirm a stochastic model in which a higher intrinsic rearrangement activity of the κ over the λ gene loci appears to be responsible for the high κ/λ ratio found in normal mice.

Secondary rearrangements can in principle occur at several Ig loci. Thus far, two examples of Vh-gene replacement in which an already rearranged VhDjķ-rearranged H chain locus could be altered with a second Vh gene have been described (43, 44). The κL chain locus has a series of Vk- and Jk- segments that allow subsequent rearrangements in an already rearranged locus by inversion or deletion of intervening sequences (45-48). In normal B cells, circular DNAs of rearranged VJs have been demonstrated of which a large part is in-frame and thus potentially functional (49). Moreover in the μH, κL chain-expressing NFS-5 tumor cells, λL chain rearrangement may occur and thus κL chains can be exchanged.

Figure 4. Proliferation and differentiation of bcl-2-5 pre B cells in the presence of hydroxyureas. Proliferation of bcl-2-5 pre B cells on PA6 stromal cells in the presence (●) or absence of IL-7 (△) and in the presence of different concentrations of hydroxyureas was determined by [3H]thymidine uptake as described (23). The expression of slgM was determined at day 3 of culture on PA6 stromal cells without IL-7 and in the presence of different concentrations of hydroxyureas as described (23). Similar results were obtained with other (fetal liver- or bone marrow-derived) Eμ-bcl-2 pre-B cell lines.
by λL chains (50). Hence, the rearrangement machinery of a B cell does not automatically stop after deposition of a complete Ig in the cell membrane.

Secondary rearrangements, also termed "receptor editing" (51, 52) could either silence unwanted specificities and/or salvage B cells with new desirable specificities. Recent studies (51, 52) using mice expressing an autoantibody as a transgene have shown that immature B cells in the bone marrow of these mice can be rescued after encounter with their autoantigen by exchanging the transgenic L chain in the Ig molecule with an endogenously encoded L chain. This L chain replacement is possible because these immature B cells still express RAG-1 and RAG-2 (51). On the other hand, crosslinking of slg on immature B cell lines from N-myc-tg mice which express slg as well as RAG-1 and RAG-2 simultaneously has been found to lead to a rapid and reversible down-regulation of RAG-1 and RAG-2 expression (53). Therefore, occupancy of slg on (immature?) B cells can either downregulate (at least in N-myc-tg mice), or preserve (at least in autoantibody-tg mice) the expression of RAG-1 and RAG-2 and, consequently, the capacity to express another L chain. This indicates that the signals given to an immature B cell via slg, as in mature B cells, can result in different reactions of these cells. It remains to be seen how the mode of Ig-receptor occupancy, additional cell contacts with the antigen-presenting environment, and secondary signals by cytokines influence these reactions.

Ongoing Ig gene rearrangements in slg + B cells are likely to generate B cells with more than one antibody specificity. In practice, this may be avoided by the rapid apoptosis of the immature B cells which leaves little time for secondary rearrangements, before the cells die, or before they are selected into the peripheral pool of mature B cells. This might also be the reason for the fact that the rescued peripheral B cell pool in mice with transgenic H and L chain genes encoding an autoantibody is relatively small (51). These scenarios of early B cell development are reminiscent of similar stages of T cell development, where immature α/β TcR + thymocytes keep expressing RAG-1 and RAG-2 and continue to rearrange α chain gene loci until they are positively selected into the pool of MHC-restricted CD4 + or CD8 + T cells (54, 55).

Immature lymphocytes from Eμ-bcl-2-tg mice of both T and B cell lineages appear suitable target cells to study the cellular and molecular requirements of positive selection into the peripheral, long-lived pools of cells.

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