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*J Immunol* 1999; 162:1931-1940; ;
http://www.jimmunol.org/content/162/4/1931

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Regulation of Cell Survival During B Lymphopoiesis: Apoptosis and Bcl-2/Bax Content of Precursor B Cells in Bone Marrow of Mice with Altered Expression of IL-7 and Recombinase-Activating Gene-2

Liwei Lu, Prosanto Chaudhury, and Dennis G. Osmond

B cell development in mouse bone marrow depends critically upon IL-7. To examine the possible in vivo trophic role of IL-7, we have quantitated apoptosis and Bcl-2 family proteins in populations of phenotypically defined B lineage cells in IL-7-deficient and IL-7-overexpressing mice. Using immunofluorescence labeling, multiparameter flow cytometry, and a short-term culture assay, we show that the apoptotic rates of precursor B cells, but not of more mature B cells, are enhanced by IL-7 gene deletion, associated with increased intracellular content of Bax and decreased Bcl-2, while, conversely, an IL-7 transgene suppresses precursor B cell apoptosis and produces low Bax and high Bcl-2 levels. During normal B cell development, high Bax/Bcl-2 ratios characterize cells undergoing greatest apoptotic cell death. Pro-B cells in RAG-2-/- mice, all destined to abort, show elevated Bax levels and Bax/Bcl-2 ratios. By comparison with the elevated rate of pro-B cell apoptosis in RAG-2-/- mice, provisional estimates have been made for the fraction of pro-B cells undergoing apoptosis in normal mice (70%), IL-7-/- mice (85%), and IL-7 transgenic mice (35%). The results demonstrate that IL-7 strongly promotes in vivo cell survival and maintains antiapoptotic Bcl-2/Bax ratios during the development of precursor B cells in mouse bone marrow. The Journal of Immunology, 1999, 162: 1931–1940.
B cell lines rapidly undergo apoptotic cell death following IL-7 withdrawal (22, 23). However, the possible role of IL-7/IL-7R engagement in regulating precursor B cell survival in vivo is controversial. While the inhibition of B cell development in mice lacking either IL-7 or IL-7R would be consistent with a much reduced cell survival of precursor B cells, it might, alternatively, reflect simply an absence of proliferative expansion (13, 24).

An indication of the relationship between IgM assembly, IL-7 stimulation, and precursor B cell survival may be provided by effects on apoptosis-regulatory proteins, notably the Bcl-2 family. These intracellular membrane-bound proteins consist of both proapoptotic and antiapoptotic members, interacting to form homodimers and heterodimers (25). The ratio between antiapoptotic Bcl-2 and proapoptotic Bax, as well as between other Bcl-2 homologues, is critical in determining the survival of many cell types (26–28). While this ratio is inherent to stages of cell development, it can be reset by either endogenous or external stimuli (29–31). In vitro evidence suggests that such a mechanism may be involved in IL-7 signaling: IL-7 withdrawal is followed by reduced Bcl-2 expression, while added IL-7 promotes cell survival and induces Bcl-2 (32, 33). In vivo, the enforced expression of Bcl-2 by a transgene promotes survival of pro-B cells in BM of normal, RAG-1−/− and SCID mice (34–36), but not, apparently, in IL-7−/− mice or cytokine γ-chain-deficient mice (37, 38). Thus, the in vivo possible relationships between IL-7 stimulation, Bcl-2/Bax ratio, and IgM expression in determining the survival of precursor B cells remain to be verified.

To examine the hypothesis that IL-7 plays an important role in determining in vivo survival of precursor B cells in mouse BM mediated by Bcl-2 family proteins, we have now directly measured rates of apoptosis and the cellular content of Bcl-2 and Bax proteins during B cell differentiation under conditions of absence and excess of IL-7. Immunofluorescence labeling and multiparameter flow cytometry have been used to quantify phenotypic stages of B cell development, the incidence of apoptotic cells at each stage ex vivo, and their rate of accumulation during short-term culture assays, in which apoptotic cells are not removed by macrophages (4). An extreme range of in vivo IL-7 levels has been provided by using IL-7−/− mice (13) and IL-7 Tg mice (39), respectively. To examine the involvement of Bcl-2 family proteins in survival of precursor B cells in vivo, we have compared the Bax/Bcl-2 protein ratio by flow cytometry at successive stages of B cell development in normal, IL-7−/−, IL-7 Tg, and RAG-2−/− mice. The results reveal that apoptosis of precursor B cells is enhanced by IL-7 gene deletion and inhibited by IL-7 Tg expression, associated with reciprocal alterations in Bax/Bcl-2 ratio. Pro-B cells in RAG-2−/− mice similarly show an association between elevated apoptosis and altered Bcl-2/Bax ratios, while also providing estimates of the apoptotic fractions in IL-7−/− and IL-7 Tg mice.

Materials and Methods

**Mice**

Male C3H/HeJ and RAG-2−/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-7 Tg mice were provided by Dr. R. Cerédig (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France). They were generated by backcrossing C57BL/6 × DBA2J mice, carrying a high copy number of murine IL-7 cDNA under the control of MHC class II (I-E) promoter to C57BL/6 mice (39). Age- and strain-matched normal mice served as controls. Homozygous IL-7−/− mice and wild-type controls (IL-7+/+) were provided by Drs. R. Murray and U. von Freeden-Jeffry (DNAX, Palo Alto, CA) (13). All mice were used at 8–12 wk of age.

**BM cell suspensions and culture**

Femoral BM cells were flushed and pooled from groups of three to five mice, as described (4). Cell suspensions were adjusted to 40 × 10^6 cells/ml in MEM with 1-glutamine (Life Technologies, Grand Island, NY), supplemented with heat-inactivated 10% (v/v) newborn calf serum (Life Technologies). Samples were either processed immediately or cultured at 37°C in a humidified incubator with 5% CO_2 for 4 h.

**Phenotypic labeling**

BM cell samples were surface labeled with phycoerythrin-conjugated anti-B220 (RA3-6B2; PharMingen, San Diego, CA) and anti-IgM (Southern Biotechnology Associates, Birmingham, AL), as described (4). To examine both cell surface and intracytoplasmic μ-chain expression (total μ), cells were fixed with cold 70% ethanol (4) and stained with biotin-conjugated anti-μ-chain mAb (Southern Biotechnology Associates), revealed by streptavidin-Red 670 (Life Technologies).

**Panning separation of BM B lineage cells**

BM cell suspensions (1 × 10^6 cells) were evenly placed in anti-B220-coated petri dishes and incubated at 4°C for 40 min, as described (4). After thorough rinsing with cold PBS, adherent B220+ B lineage cells were recovered by gentle scraping, transferred to anti-IgM mAb-coated dishes, and incubated at 4°C for 40 min. Finally, the nonadherent B220+ slgM− precursor B cells and adherent slgM+ B cells were separately collected. Sample purity was in each case verified by flow cytometry.

**Immunoblotting analysis**

Lysates from fresh panning-purified B220+ slgM− precursor B cells and slgM+ B lymphocytes were prepared by incubation in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-ME, and 0.01% bromophenol blue. Sample proteins were separated on 12% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes (0.45 μm; Pierce, Rockford, IL). After blocking with 5% skim milk in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 1 h at room temperature, the membranes were incubated first with either hamster anti-Bcl-2 Ab (PharMingen) or rabbit anti-Bax Ab (Oncogene Research Products, Cambridge, MA) at 1 μg/ml in blocking buffer and then with either horse radish peroxidase-conjugated goat anti-hamster IgG or horseradish peroxidase anti-rabbit IgG Abs (Jackson Immunoresearch, West Grove, PA), respectively. Finally, proteins were revealed with an enhanced chemiluminescence ECL system (Amersham Life Science, Buckinghamshire, U.K.), according to the manufacturer’s instructions.

**Cell sorting**

Samples of pooled BM cell suspensions from groups of five mice were immunolabeled for B220 and IgM expression. Large and small B220+ slgM− precursor B cells were purified according to forward scatter scale using a FACS (FACS Vantage; Becton Dickinson, Mountain View, CA) at 1 μg/ml in blocking buffer and then with either horseradish peroxidase-conjugated goat anti-hamster IgG or horseradish peroxidase anti-rabbit IgG Abs (Jackson Immunoresearch, West Grove, PA), respectively. Finally, proteins were revealed with an enhanced chemoluminescence ECL system (Amersham Life Science, Buckinghamshire, U.K.), according to the manufacturer’s instructions.

**Apoptic assay**

After phenotypic labeling, ethanol-fixed cells were suspended in 50 μg/ml RNase (Boehringer Mannheim, Mannheim, Germany) and 50 μg/ml propidium iodide (Sigma) in PBS and kept on ice in the dark until analysis, as described (4).

**Immunofluorescent staining of intracellular Bcl-2 and Bax protein**

Phenotypically labeled cells were incubated with either hamster anti-Bcl-2 mAb (PharMingen) or rabbit anti-Bax Ab (Oncogene Research Products) at 1/50 dilution in PBS with 0.1% saponin for 30 min on ice, followed by either FITC goat anti-hamster IgG Ab (Cedarlane Laboratories Limited, Ontario, Canada) or goat anti-rabbit IgG Ab (Southern Biotechnology Associates) at 1/50 dilution in PBS, respectively. Samples stained with either FITC hamster IgG or FITC rabbit IgG isotype control Abs (Cedarlane Laboratories Limited) were used to determine fluorescence background.

**Flow-cytometric analysis**

Immunostained cells were analyzed with a flow cytometer (FACScan; Becton Dickinson) using Lysys II software. A minimum of 10,000 B lineage cells of defined phenotype was analyzed from each sample. Cell debris and clumps were excluded by setting a gate on forward scatter versus side scatter. Apoptotic cells were identified in the hypodiploid region of DNA.
content profiles, as described (4). Cellular content of Bcl-2 and Bax protein was measured as the mean fluorescence intensity of defined subpopulations of B lineage cells.

**Statistics**

Statistical analysis was performed using Student’s t test. A value of p < 0.05 was considered significant.

**Results**

**Expression of Bcl-2 and Bax proteins during normal B lymphopoiesis**

We have previously compared the rates at which precursor B cells undergo apoptosis at successive stages of differentiation in mouse BM (4). We now examine the corresponding expression of Bcl-2 and Bax proteins by precursor B cells and then evaluate the capacity of IL-7 to modify both in vivo cell survival and expression of Bcl-2 and Bax. First, whole cell lysates from panning-purified B220^+ sIgM^− precursor B cells and sIgM^+ B lymphocytes were examined by Western blot analysis. As shown in Fig. 1A, the 26-kDa Bcl-2 protein was constitutively expressed by B lineage cells in BM. However, B220^+ sIgM^− precursor B cells expressed much more Bcl-2 than sIgM^+ B lymphocytes. Precursor B cells also expressed a higher level of Bax than B lymphocytes (Fig. 1B). In contrast to BM B lymphocytes, sIgM^+ cells in the spleen displayed a relatively large amount of Bcl-2, but barely detectable Bax (Fig. 1, A and B).

To examine the developmentally regulated expression of Bcl-2 and Bax at the single cell level, the Bcl-2 and Bax protein content of B lineage cells was examined by three-color flow cytometry (Fig. 2). Starting from a moderately high mean level of Bcl-2 protein per cell among B220^+ μ^− pre-B cells, the Bcl-2 content increased further at the large cμ^− sμ^− pre-B cell stage, subsequently falling to low levels among small cμ^− sμ^− pre-B cells and sIgM^+ B lymphocytes (Figs. 2 and 3A). The lowest Bcl-2 levels were exhibited by immature sIgMlow B lymphocytes. Bax protein levels followed a similar pattern during B cell differentiation, but fluctuated more widely (Figs. 2 and 3B). The mean Bax content per cell increased markedly from the B220^+ μ^− pre-B cell population to cμ^− sμ^− pre-B cells, dropping abruptly to only one-third this level among cμ^− sμ^− pre-B cells, sIgMlow, and sIgMhigh cell populations (Fig. 3B).

Because the ratio of the proapoptotic protein, Bax, to the antiapoptotic protein, Bcl-2, is more important in governing cell survival than the level of either protein alone (28), we examined this ratio at each phenotypically defined stage of B cell development (Fig. 3C). Although the mean fluorescence intensity of cells immunolabeled for Bcl-2 and Bax gives only a relative measurement of the protein content per cell, a comparison of the mean fluorescence intensity of cells under identical analytical conditions provides a comparison of mean protein levels. Two precursor B cell populations had higher Bax to Bcl-2 protein ratios than the rest (p < 0.01; Fig. 3C). These were the large cμ^− sμ^− pre-B cells and sIgM^+ low immute B lymphocytes. In contrast, the lowest Bax/Bcl-2 ratios were those of small cμ^− sμ^− pre-B cells and mature sIgM^high B lymphocytes. Thus, the data suggest that Bax, Bcl-2, and the Bax/Bcl-2 ratio are developmentally regulated during B cell differentiation. In particular, the stages of high Bax/Bcl-2 ratio coincide with stages previously shown to be associated with high rates of apoptotic cell death, viz cells at the pro-B/pre-B transition and immature B lymphocytes just beginning to express sIgM (4).

**Altered B cell development in IL-7-deficient and IL-7 Tg mouse BM**

IL-7−/− mice and IL-7 Tg mice provided models to examine the effect of IL-7 on in vivo apoptosis and Bax/Bcl-2 ratios during B cell development. We first quantitated the B lineage populations in BM to supplement previous studies that have used different phenotypic criteria or analytical methods (13). A critical step in early B cell development is synthesis of the μ-chain, signaling transition from the pro-B cell stage of V_{H}D_{H}J_{H} rearrangement to the pre-B cell expressing pre-BCR (3). While various cell surface markers correspond approximately to the stage of pro-B cells (CD43^+ , c-kit^+ ) and pre-B cells (CD43^− , CD25^+ ), respectively (5–7, 40),
their correlation with \(\mu\)-chain expression is indirect and inexact (7). Many \(c\mu^+\) pre-B cells lack CD25 (41). Hence, we have examined intracytoplasmic \(\mu\)-chains directly by immunolabeling and flow cytometry of fixed, permeabilized cells (4, 7) (Fig. 4). In IL-7 \(^/-\) mice, the total BM cellularity was reduced to about 65% that of controls, attributable mainly to lack of B lineage cells. Only 7.6 \(\pm\) 1.2% B220 \(^+\) cells were detected, compared with 35 \(\pm\) 2.1% in controls. The incidence of B220 \(^+\) \(\mu^+\) pro-B cells, lacking both \(c\mu\) and \(s\mu\), was within the normal range, but both \(c\mu^+s\mu^-\) pre-B cells and \(sIgM^-\) B cells were reduced to about one-tenth normal incidence and absolute number (Fig. 4A). Thus, B cell development was profoundly inhibited, although not completely eliminated, at the pro-B/pre-B transition. These findings are in general agreement with flow-cytometric measurements of cell frequencies based on cell surface criteria (13), and, in addition, provide values for the actual number of cells in each precursor B cell population.

In marked contrast with IL-7 \(^/-\) mice, both B220 \(^+\) \(c\mu^+s\mu^-\) pre-B cells and \(c\mu^+s\mu^+\) pre-B cells of IL-7 Tg mice were greatly increased in numbers (Fig. 4B). \(sIgM^-\) B lymphocytes were also augmented, although to a lesser extent. The data are consistent with a previous report on IL-7 Tg mice based on epifluorescence microscopy of cytospun cells, revealing pronounced hyperplasia of the B cell lineage in BM (17).

**Enhanced rate of precursor B cell apoptosis in IL-7-deficient mice**

Apoptosis of B lineage cells was evaluated by the criterion of hypodiploid DNA content, analyzed by flow cytometry, as described (4) (Fig. 5). In freshly prepared BM cell suspensions from IL-7 \(^/-\) mice, the incidence of apoptosis among B220 \(^0\) \(sIgM^-\) precursor B cells (2.6 \(\pm\) 0.2%) was consistently somewhat higher \((p < 0.05)\) than that in controls (2.1 \(\pm\) 0.4%). In contrast, the few surviving \(sIgM^-\) B lymphocytes in IL-7 \(^/-\) mice showed essentially the same apoptotic incidence (1.3 \(\pm\) 0.4%) as in normal mice (1.2 \(\pm\) 0.2%). The hypodiploid B cells detected ex vivo represent the number of apoptotic cells present at any given time in BM between the onset of apoptosis and their ingestion by macrophages (1, 9). Because of the rapidity of this clearance, even modest changes in apoptotic index, as seen in the precursor B cells of IL-7 \(^/-\) mice, may represent substantial differences in the fraction of cells actually undergoing apoptosis. To provide a more sensitive assay of the kinetics of B cell apoptosis, a short-term culture system was used to reveal the rate at which apoptotic cells accumulate without macrophage-mediated deletion (4) (Figs. 5 and 6). This system amplifies the small differences in apoptotic index seen in
fresh BM samples. During the culture period, the apoptotic incidence of B220<sup>+</sup>sIgM<sup>-</sup> precursor B cells from IL-7<sup>−/−</sup> mice increased steeply in linear fashion, to reach 22.5 ± 1.8% by 4 h (Fig. 6), a much higher rate than B220<sup>+</sup>sIgM<sup>-</sup> precursors from normal mice (13.5 ± 1.6% by 4 h). In contrast, the small population of sIgM<sup>+</sup>B lymphocytes from IL-7<sup>−/−</sup> mice showed essentially normal apoptotic kinetics (Fig. 6).

These findings suggest that deficiency of IL-7 results in a substantially elevated rate of apoptosis by precursor B cells (5 ± 0.4%/h vs 2.9 ± 0.3%/h), but not by B lymphocytes under the conditions of short-term culture. To some extent, this result might have reflected the different composition of B220<sup>+</sup>sIgM<sup>-</sup> precursor B cells, consisting predominantly of pro-B cells (80%) in IL-7<sup>−/−</sup> mice and a minority of pro-B cells (35–45%) together with pre-B cells in normal mice. However, since the apoptotic rate of B220<sup>+</sup>µ<sup>-</sup> pro-B cells normally closely resembles the mean apoptotic rate of large and small c<sup>+</sup>sµ<sup>−</sup> pre-B cells combined (4), this factor cannot account for the marked changes observed in IL-7<sup>−/−</sup> mice. This point was further tested in IL-7 Tg mice, in which B220<sup>+</sup>sIgM<sup>-</sup> cells and sIgM<sup>+</sup> cells, on the other hand, displayed normal apoptotic incidences, both before and after culture (Fig. 6).

Taken together with the findings in IL-7<sup>−/−</sup> mice, the data indicate that the rate of apoptosis among large B220<sup>+</sup>sIgM<sup>-</sup> cells, representing pro-B cells and large cycling pre-B cells, is inversely related to the in vivo level of IL-7, whereas apoptosis among later stages of B cell differentiation in BM is relatively IL-7 independent.

Reciprocal changes of Bcl-2 and Bax protein levels among precursor B cells in IL-7-deficient and IL-7 Tg mice

To determine whether the effect of IL-7 on apoptosis of early precursor B cells was associated with changes in Bcl-2 family proteins, we performed a three-color flow-cytometric analysis of the intracellular expression of Bcl-2 and Bax protein by B lineage cells. As shown in Fig. 8, among B220<sup>+</sup>sIgM<sup>-</sup> precursor B cells in freshly prepared BM samples from IL-7<sup>−/−</sup> mice, the mean Bcl-2 protein content per cell was reduced to half normal levels, while Bax protein was increased 1.5-fold. These changes substantially increased the Bax/Bcl-2 ratio. On the other hand, the mean levels of Bcl-2 and Bax among sIgM<sup>+</sup> B lymphocytes from IL-7<sup>−/−</sup> mice remained comparable with normal values.
Exactly opposite changes in Bcl-2 and Bax expression by early precursor B cells were seen in IL-7 Tg mice. The mean Bcl-2 protein content of large B220<sup>+</sup> sIgM<sup>−</sup> precursors was significantly increased compared with normal values, while Bax levels were reduced to half normal, thus effectively reducing the Bax/Bcl-2 ratio (Fig. 9). Again, however, only lesser degrees of Bax protein reduction, and no changes in Bcl-2 levels, were shown by small B220<sup>+</sup> sIgM<sup>−</sup> cells and sIgM<sup>+</sup> cells (Fig. 9).

**Pro-B cell apoptosis and Bax/Bcl-2 expression in RAG-2-deficient mice**

In BM of RAG-2<sup>−/−</sup> mice, no cμ<sup>+</sup> sμ<sup>−</sup> pre-B cells or slgM<sup>−</sup> B lymphocytes were detected (Fig. 10A). Thus, B cell development proceeded no further than the pro-B cell stage, and the B lineage cells detected in BM samples were exclusively of B220<sup>+</sup> phenotype. We used this model to provide a comparative assay by which to evaluate the degree of apoptosis induced in early precursor B cells by IL-7 deficiency.

The apoptotic incidence among B220<sup>+</sup> μ<sup>−</sup> pro-B cells was significantly greater in RAG-2<sup>−/−</sup> mice than in controls (2.7 ± 0.3% vs 2 ± 0.2%; p < 0.01). During short-term culture for 2–4 h, the apoptotic incidence among B220<sup>+</sup> μ<sup>−</sup> cell populations increased linearly. In RAG-2<sup>−/−</sup> mice, the B220<sup>+</sup> μ<sup>−</sup> pro-B cells became apoptotic at a greater rate than normal (5.8 ± 0.2%/h vs 4 ± 0.3%/h). After 4-h incubation, the hypodiploid incidence among B220<sup>+</sup> μ<sup>−</sup> pro-B cells reached 26.2 ± 1.3% in RAG-2<sup>−/−</sup> mice, but only 18.2 ± 1.6% in controls (Fig. 10B).

Intracellular Bcl-2 and Bax protein levels were evaluated by three-color flow-cytometric analysis (Fig. 10C). Bax expression by B220<sup>+</sup> μ<sup>−</sup> pro-B cells was up-regulated in RAG-2<sup>−/−</sup> mice, to almost 2.5-fold normal values. Although Bcl-2 protein content per cell remained close to normal levels, the Bax/Bcl-2 ratio showed a substantial increase, similar to that among precursor B cells in IL-7<sup>−/−</sup> mice (Fig. 8).

**Discussion**

Many interacting factors may determine whether precursor B cells survive or die in mouse BM. The present study provides evidence that IL-7 is one factor that promotes the survival of precursor B cells in vivo, in addition to its reported proliferative effects.

IL-7 gene deletion results in profound impairment in production of cμ<sup>+</sup> sμ<sup>−</sup> pre-B cells and slgM<sup>−</sup> B lymphocytes, whereas the earlier populations of B220<sup>+</sup> μ<sup>−</sup> pro-B cells remain almost unaffected (13) (Fig. 4). Ablation of either the α- or γ-chain of the IL-7R also produces a deficiency of B lymphopoiesis closely resembling that in IL-7<sup>−/−</sup> mice, but evident from an earlier pro-B cell stage (18, 21). However, such IL-7R<sup>−/−</sup> models are complicated by the fact that the common cytokine γ-chain receptor is used by other cytokines, including IL-2, IL-4, IL-9, and IL-15, while the α-chain appears also to be used by thymic stromal-derived lymphopoietin (TSLP) (21, 42). IL-7 gene-deleted mice thus provide the method of choice to address the in vivo actions of IL-7.
without at the same time functionally inactivating other cytokines. In contrast with IL-7 gene deletion, an IL-7 transgene producing constitutive overexpression of IL-7 is associated with much expansion of all precursor B cell populations (17) (Fig. 4). That the effects seen in IL-7$^{-/-}$ and IL-7 Tg mice are due, at least in part, to alterations in the fraction of surviving precursor B cells, rather than simply to altered proliferation, is now suggested by two complementary lines of evidence: the rate at which precursor B cells enter a cell death pathway and the ratio between the intracellular content of two apoptosis-regulatory proteins, Bax and Bcl-2.

In the absence of IL-7, B220$^+$sIgM$^-$ precursor B cells (A), small B220$^+$sIgM$^-$ precursor B cells (B), and sIgM$^+$ B cells (C) in freshly prepared BM cell suspensions from IL-7 Tg and normal mice. Histograms (above) are representative of three separate experiments. Protein expression (below) was measured as mean fluorescence intensity per cell, representing the respective mean values from normal mice as equal to 1 (mean ± SD).

**FIGURE 9.** Expression of Bcl-2 and Bax among large B220$^+$sIgM$^-$ precursor B cells (A), small B220$^+$sIgM$^-$ precursor B cells (B), and sIgM$^+$ B cells (C) in freshly prepared BM cell suspensions from IL-7 Tg and normal mice. Histograms (above) are representative of three separate experiments. Protein expression (below) was measured as mean fluorescence intensity per cell, representing the respective mean values from normal mice as equal to 1 (mean ± SD).

**FIGURE 10.** B cell lineage populations, apoptosis, and Bcl-2/Bax expression in RAG-2$^{-/-}$ mice: A, Flow-cytometric analysis of immunolabeled BM cell suspensions from RAG-2$^{-/-}$ and normal mice. Incidences of various defined populations of precursor B cells are indicated. B, Incidence of hypodiploid cells among B220$^+$μ$^-$ cells in BM suspensions from RAG-2$^{-/-}$ and control mice after 0–4-h incubation. Data represent three separate experiments (mean ± SD). C, Representative histograms (above) showing expression of endogenous Bcl-2 and Bax proteins among B220$^+$μ$^-$ B cells in freshly prepared BM cell suspensions from RAG-2$^{-/-}$ and normal mice. Bcl-2 and Bax expressions (below) were measured as mean fluorescence index by flow cytometry, expressed relative to respective normal values. Data derived from three separate experiments (mean ± SD).
cells, the number of apoptotic B lineage cells increases linearly with time for periods of 4 to 6 h without the cells being ingested by macrophages, as would occur in vivo (9). In view of the limited time for which the cells are removed from the body, the short periods of culture, and the linearity of the apoptotic response, the observed accumulation of apoptotic cells should represent mainly the progression of apoptosis initiated by B cell differentiation signals operating in vivo, rather than secondary factors related to the in vitro environment (4). As measured in this assay, the effect of IL-7 deprivation on in vivo lineage apoptosis is selective, the apoptotic rate being increased within compartments of precursor B cells, but not among the small number of slgM+ B lymphocytes still being produced. The results are consistent with a specific effect restricted to B lineage cells that constitutively display IL7R (43). It is most noteworthy that elevated in vivo levels of IL-7 can inhibit the normal apoptosis of precursor B cells that would otherwise eliminate unwanted or undesirable cells. To some extent, such effects might simply be attributable to fluctuations in the degree of proliferative expansion of precursor B cells, depending upon the overexpression or lack of IL-7 in vivo. Thus, it could be postulated that the substantial number of pro-B cells normally destined to undergo apoptotic selection remains unchanged, but is diluted to a lesser or greater extent by surviving precursor B cells proliferating under the influence of IL-7, resulting in only apparent changes in the apoptotic rates. This may contribute a small contributory factor in the case of IL-7−/− mice, in which B220+μ− pro-B cells form a greater proportion of the B220+slgM− phenotype (80%) than normal (35–45%). It is inadequate, however, to account for more than a fraction of the greatly enhanced apoptosis observed among B220+slgM− cells in these mice and can in no way explain the suppressed apoptosis in IL-7−/− Tg mice, in which the proportion of B220+μ− pro-B cells among B220+slgM− precursors is near normal. Hence, the data demonstrate that IL-7 gene modification in vivo can profoundly affect the survival of B220+slgM− precursors in BM, their apoptotic cell death being partially blocked by overexpression of IL-7 and enhanced by lack of IL-7.

In many cell systems, Bcl-2 and Bax proteins are involved in regulating apoptosis both during cell development and in response to a range of cell death-inducing stimuli (44). In vitro evidence from various cell systems suggests that high expression of Bcl-2 homodimers inhibits the release of protease (caspase) activators from mitochondria, while greater Bax expression leading to the formation of Bcl-2/Bax heterodimers and Bax/Bax homodimers allows caspase activators to enter the cytosol, and thus to initiate the apoptotic effector cascade (25–28). Intracellular levels of Bax and Bcl-2, and especially the ratio between them, can thus provide an index of susceptibility to apoptosis (28). The present results show that changes in mean Bax:Bcl-2 ratio correlate with changes in the apoptotic fraction of defined precursor B cell populations during normal B lymphopoiesis. The Bax:Bcl-2 ratio is highest at the two stages of B cell development in BM when apoptosis is known to be most marked (4). This suggests that changes in expression of Bcl-2 family proteins are involved in mediating apoptosis during the normal selection of precursor B cells in BM. This accords with the effects of enforced expression of bcl-2 by a transgene that results in expanded populations of precursor B cells in BM (34, 36), reflecting an increase in their survival fraction.

We now show in IL-7−/− and IL-7 Tg mice that the mean Bax:Bcl-2 ratio among precursor B cells in BM is inversely proportional to in vivo levels of IL-7, a selective effect not seen among more mature slgM− B lymphocytes. This is consistent with the effect of IL-7 levels on apoptotic rates, determined independently. Similar effects have been reported in vitro (22, 23). Precursor B cells removed from stromal cell cultures increase Bax expression and down-regulate Bcl-2 mRNA, correlating with an initiation of apoptosis (29). The IL-7 Tg mice used in the present work carry an IL-7 transgene under the control of the MHC class II promoter (38). Thus, in addition to the constitutive expression of IL-7 by stromal cells, some immature slgM− B cells as they begin to express MHC II will produce IL-7 under the influence of the MHC class II promoter, and thus be subject to autocrine IL-7 stimulation (17). This may account for the slight changes in B expression and apoptosis seen in these cells in addition to the major changes in the precursor B cell compartment. Collectively, the data demonstrate that gene modifications that alter IL-7 levels in vivo mark different stages of B cell development in BM (34, 36), reflecting an increase in their survival fraction.

While IL-7 greatly modifies the apoptosis of precursor B cells at the pro-B/pre-B transition stage in vivo, it appears not to be an absolute requirement for precursor B cell survival. Even in IL-7−/− mice, one-tenth of the normal numbers of viable B lymphocytes displaying essentially normal apoptotic rates still appear in BM and accumulate in the periphery. These may represent a minor subpopulation of B lineage cells that are IL-7 independent with respect to their survival, possibly responding to compensatory factors for which TSLP could be a candidate (21, 42). The identity of such IL-7−/−-independent survival signals remains to be verified. In RAG-2−/− mice, in contrast, no cells progress beyond the pro-B cell stage. This emphasizes the overriding importance of a functional Ig μ heavy chain for precursor B cell survival and makes the RAG-deficient mouse an attractive model for estimating the fraction of cells undergoing apoptosis at this stage of B cell development in both normal and perturbed states.

Individual cells entering the B220+μ− pro-B cell stage of differentiation may be visualized as remaining in that phenotypic compartment for a certain length of time (transit time) and then leaving the compartment by either expressing μ-chains, thus proceeding into the compartment of pre-B cells, or dying by apoptosis. From the observed rate of apoptosis among B220+μ− pro-B cells in RAG-2−/− mice (5.8 ± 0.2%/h), it may be calculated that 100% of cells in the compartment would become apoptotic in a period of 17.2 h. Knowing that all of these cells are indeed destined to become apoptotic, 17.2 h represents the mean transit time of cells in the B220+μ− pro-B cell compartment in RAG-2−/− mice.

From the RAG-2−/− model, the fraction of pro-B cells undergoing apoptosis in normal BM can be derived. Assuming that the differentiation kinetics and mean transit times of pro-B cells are identical in normal and RAG-2−/− mice, as suggested by their closely similar cell population sizes and proliferative activity, a comparison of the apoptotic rates of B220+μ− cells in RAG-2−/− mice and normal mice (4 ± 0.3%/h) suggests that 70% of B220+μ− cells are deleted by apoptosis even in normal BM. Although subject to the premises inherent in the calculation, this value is consistent with estimates of both the normal frequency of nonfunctional Ig H chain gene rearrangements that may be expected to result in apoptosis (2, 3, 45) and the fraction of normal precursor B cells previously calculated to enter apoptosis based on cell population dynamics and apoptotic rates during B lymphopoiesis (4). The present data thus provide further evidence for the large scale on which precursor B cells are deleted in normal mouse BM.
From comparisons between the apoptotic rate of precursor B cells from RAG-22/2 and IL-7R2/2 mice (5 ± 0.4%/h), it may be calculated that the fraction of precursor B cells undergoing apoptosis in the absence of IL-7 is increased to 85%. Since, as noted, the precursor B cell populations as monitored in IL-7R2/2 mice include a number of pre-B cells, in addition to the predominance of pro-B cells, this is probably a somewhat conservative estimate of the apoptotic fraction. On the other hand, assays in IL-7 Tg mice of large B220+ IgM+ precursor B cells, representing cells just before and after the pro-B/pre-B transition point, reveal a halving of the normal apoptotic rate, equivalent to an apoptotic fraction of 35%. Thus, even with excessive IL-7 stimulation sufficient to produce striking expansion of B lymphopoiesis, the fraction of precursor B cells undergoing apoptosis, although much reduced, is still substantial. The foregoing values for apoptotic fractions represent initial estimates requiring further verification. Nevertheless, they clearly show that IL-7 plays a powerful role in promoting precursor B cell survival in mouse BM. Yet, unlike the expression they clearly show that IL-7 plays a powerful role in promoting precursor B cell survival in mouse BM. Yet, unlike the expression 

Our data documenting the influence of IL-7 and Bcl-2 family proteins on precursor B cells have remarkable similarity to effects on the early T cell lineage. IL-7 gene deletion inhibits T cell development associated with a reduced Bcl-2 expression in early thymocytes both before and during the stage of TCR rearrangement (49), rescued by a Bcl-2 transgene (38). Bcl-2 gene deletion is associated with disappearance of T cells (50). T cells from aging humans showed increased Bax, decreased Bcl-2, and enhanced susceptibility to apoptosis (51). On the other hand, IL-7 protects human T cells from apoptosis induction and inhibits the associated down-regulation of Bcl-2 (21). Thus, IL-7 may provide a survival signal via Bcl-2 family proteins to both T cells in thymus and B cells in BM at equivalent stages of cell development when the respective Ag-binding receptors are being generated and tested. The ability of IL-7 to promote survival at the pro-B/pre-B cell transition in BM may have important implications for B cell dysregulation, notably neoplasia. Many IL-7 Tg mice develop malignant lymphomas of early B lineage phenotype (17, 39). Such oncogenesis might be initiated among early precursor B cells in BM. Cells that sustain genetic errors, including chromosomal translocations during Ig gene recombination, and that normally would undergo apoptotic selection at the pro-B/pre-B cell transition, may be driven by high levels of IL-7 to survive past this checkpoint. Further genetic errors occurring subsequently in some such aberrant cells could result in oligoclonal or monoclonal B lineage tumors. Thus, the enhancing effect of IL-7 on precursor B cell survival in conjunction with its proliferative stimulation and possible overstimulation of VH DJH recombination (22) could help to explain the association between prolonged hyperplasia of pro-B cells and initiation of B cell neoplasms. Such prelymphomatous hyperplasia may be produced by conditions other than an IL-7 transgene (1, 52). Prolonged systemic activation of macrophages, as occurs in chronic granulomatata and malarial infection, is associated with both elevated pro-B cell proliferation and the development of B cell tumors (53). The proliferative effect appears to be mediated by circulating macrophage-derived factors acting via receptors on BM stromal cells that in turn are presumed to be induced to secrete short-range cytokines, including IL-7 (54). Increased IL-7-driven production of B lymphocytes in BM may be achieved at the cost of a less efficient quality control, enforcing the viability of precursor B cells and the dissemination of some potentially pre-neoplastic B cells. Within the milieu of mouse BM, the survival of precursor B cells is determined by a complex combination of intracellular signals and interactions with microenvironmental molecules, among which IL-7 appears to play a particularly prominent role.

Acknowledgments

We thank Dr. Rodri Ceredig (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France) for providing breeding stock of IL-7 transgenic mice (39) and Drs. Richard Murray and Ursula von-Freeden-Jeffry (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA; supported by Schering-Plough) for providing breeding stock of IL-7 gene-deleted mice (13).

References