Increased apoptosis of bone marrow pre-B cells in old mice associated with their low number

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Abstract

The number of bone marrow pre-B cells is significantly lower in 18- than in 2-month-old BALB/c mice. The percentage of apoptotic pre-B cells, freshly isolated or cultured, from 18-month-old mice was significantly greater than from 2-month-old mice. The increased percentage of apoptotic pre-B cells from old mice was associated with a decreased level of bcl-xL mRNA, detected by RT-PCR, and of Bcl-xL protein, detected by intracellular staining. Consistent with an age-associated increase in apoptosis in pre-B cells was the fact that significantly fewer pre-B cells were generated after in vitro cultures of pro-B cells from old as compared to young mice. Furthermore, fewer pre-B cells survived and fewer slg-expressing B cells were generated in cultures of pre-B cells from old as compared to young mice. In addition, there was no detectable difference in the secretion of IL-7 by bone marrow cells from 2- or 18-month-old mice. Thus, increased apoptosis of bone marrow pre-B cells in old mice appears to contribute to their decreased number.

Introduction

There is general agreement that the number of pre-B cells in bone marrow declines with age in both humans and other mammals (1–3). B cell development is known to depend on stromal cells and their cytokines, one of the most critical being IL-7 (4,5). Hardy et al. have shown that IL-7 stimulates primarily pro-B cells (6,7), although other studies suggest that IL-7 is also needed for the proliferation and/or differentiation of pre-B cells (8,9). For this reason, it has been postulated that the generation of pre-B cells in old animals might be impaired because of decreased production of or response to IL-7 (9–13).

The age-associated decline in the number of murine pre-B, B220+CD43−slg− cells, begins at 4 months of age (14). By 24 months of age other changes in bone marrow cell function have been noted (14,15). These include an impaired capacity of bone marrow stromal cells to support the proliferation of pro-B, B220+CD43−slg− cells and an impaired response of bone marrow pro-B cells to IL-7 (15). These events may contribute to the decreased number of pre-B cells seen at 24 months of age. Whether these mechanisms explain the decreased number of pre-B cells in younger mice is unknown.

In this study, we show that bone marrow pre-B cells from 18-month-old mice express a reduced level of Bcl-xL, had a higher percentage of apoptotic cells and had a lower survival in culture compared to 2-month-old mice. In contrast, there was no detectable difference in production of IL-7 by bone marrow cells from 18-month-old mice. We conclude that increased apoptosis of bone marrow pre-B cells contributes to the decreased number of these cells in 18-month-old mice.

Methods

Mice

Two- and 18-month-old female BALB/c mice were purchased from the NIA aging colony (Charles River Laboratories, Wilmington, MA) and maintained at the Cornell University Medical College Animal Resource Facilities in laminar flow hoods.

Bone marrow cells

Bone marrow cell suspensions were prepared from femurs by flushing the marrow cavity with HBSS (Sigma, St Louis,
**Apoptosis of pre-B cells**

<table>
<thead>
<tr>
<th>Table 1. The number of bone marrow pre-B cells is decreased in 18-month-old mice compared to 2-month-old mice</th>
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<tr>
<td>Age of mice (months)</td>
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<tr>
<td>CD43&lt;sup&gt;+&lt;/sup&gt;B220&lt;sup&gt;+&lt;/sup&gt;slg&lt;sup&gt;−&lt;/sup&gt;</td>
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<tr>
<td>2 (5)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>18 (5)&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Bone marrow cell subset analysis was performed by three-color flow cytometry using antibodies to murine CD43, B220 and Ig. The number of cells from a given subset was derived by direct microscopic counting of total cell numbers and cytometric measurement of the percentage comprising that subset and is given in millions per femur.

<sup>b</sup>The number of mice studied is given in parentheses.

<sup>c</sup>P < 0.01 from 2- and 18-month-old animals.

<table>
<thead>
<tr>
<th>Table 2. Bone marrow adherent cells from 2- and 18-month-old mice secrete comparable amounts of IL-7</th>
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<tr>
<td>Age of mice (months)</td>
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<tr>
<td>Mean ± SD</td>
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<tr>
<td>2 (5)&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Adherent bone marrow cells (2 × 10<sup>6</sup>) were cultured in 1 ml of complete medium for 3 days. The supernatant medium was then collected and assayed for IL-7 by ELISA.

<sup>b</sup>The number of mice studied is given in parentheses. The results are from three independent experiments.

<table>
<thead>
<tr>
<th>Table 3. Decreased survival of pre-B cells from 18-month-old mice in culture</th>
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<tr>
<td>Age of cell donors (months)</td>
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<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Pro-B</td>
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<td>2 (5)&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>pre-B</td>
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<td>18 (5)&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>pre-B</td>
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<sup>a</sup>Pro-, pre- and B cells are defined as CD43<sup>+</sup>B220<sup>+</sup>slg<sup>−</sup>, CD43<sup>+</sup>B220<sup>−</sup>slg<sup>−</sup> and CD43<sup>+</sup>B220<sup>−</sup>sg<sup>+</sup> cells respectively.

<sup>b</sup>The number of mice studied is given in parentheses.

<sup>c</sup>P < 0.05.

<sup>d</sup>P < 0.01.

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<tr>
<th>Table 4. The rate of apoptosis is increased in cultured bone marrow pre-B cells from 18-month-old mice</th>
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<tr>
<td>Age of mice (months)</td>
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<tr>
<td>2</td>
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<sup>a</sup>Freshly sorted bone marrow pre-B cells were incubated with annexin V-biotin followed by streptavidin-PE and analyzed by flow cytometry. The data are given in percentages.

<sup>b</sup>Bone marrow cells were depleted of Ig<sup>+</sup> and CD43<sup>+</sup> and cultured for 3 days. At the end of culture, CD43<sup>−</sup> cells were again depleted and the percentage of apoptotic pre-B cells was tested using antibodies to surface B220 and Ig and enzymatic labeling of 3′-OH ends of DNA strands.

<sup>c</sup>The number of mice studied is given in parentheses.

<sup>d</sup>P < 0.01 from 2- and 18-month-old mice.

**Flow cytometry**

Bone marrow cells were resuspended in HBSS without phenol red (Life Technologies, Grand Island, NY) containing 0.1% sodium azide (Sigma) and 5% FCS (Sigma) at a concentration 10<sup>6</sup>/ml. In a three-color flow cytometry analysis, 0.5 × 10<sup>6</sup> cells were incubated with 0.4 μg Cy-Chrome-labeled anti-mouse B220 antibody (PharMingen, San Diego, CA), 1 μg of FITC-labeled anti-mouse IgM/IgG/IgA heavy and light chain-specific antibody (Accurate, Westbury, NY), and 1 μg of anti-mouse biotinylated CD43 antibody (PharMingen) followed by incubation with phycoerythrin (PE)-labeled streptavidin (Accurate). After incubation, cells were washed 3 times in HBSS containing 0.1% sodium azide, 5% FCS and fixed in 1% paraformaldehyde (Sigma) HBSS solution. Cells were analyzed using FACSscan (Becton Dickinson) and CellQuest software; 10<sup>4</sup> of electronically gated cells, based on the forward/side scatter pattern, were analyzed per sample. Absolute numbers were derived by direct microscopic counting of total cell numbers and cytometric measurement of the percentage comprising the subset.

**Cell sorting**

Lymphoid cells were separated from total bone marrow cells by gradient centrifugation on Lympholyte-M (Accurate, Westbury, NY), and the percentage of apoptotic pre-B cells was tested using antibodies to surface B220 and Ig and enzymatic labeling of 3′-OH ends of DNA strands.

**Culture conditions**

Unfractionated bone marrow cells were cultured in RPMI 1640 medium (JRH, Lenexa, KS) supplemented with 10% FCS (Sigma), 2 mM L-glutamine (Whittaker, Walkersville, MA), 10 mM HEPES (Sigma), penicillin-streptomycin (Life Technologies, Grand Island, NY) and 5 × 10<sup>−5</sup> M 2-mercaptoethanol (Sigma). In the survival assay of B cell precursors, sorted...
Fig. 1. The percentage of bone marrow pre-B cells is decreased in 18-month-old mice. Bone marrow cells from 2- and 18-month-old mice were stained with antibodies to sIg–FITC (FL1), B220–Cy-Chrome (FL3) and CD43–biotin with streptavidin–PE (FL2). Intact cells selected on forward/side scatter were gated for sIg– (FL1–) cells (lower panel). The number in each square represents the percentage within each subset.

pro- and pre-B cells were cultured on stromal S17 cells for 4 days. Under these conditions, the amount of IL-7 in the supernatant medium, presumably secreted by S17 cells, was 2 ng/ml.

**Immunoassay for secreted IL-7**

Supernatant was collected from 3 day cultures of $2 \times 10^6$ adherent bone marrow cells from 2- or 18-month-old mice as described above. Ninety-six-well plates (Nunc, Roskilde, Denmark) were coated with purified mAb to murine IL-7 (Genzyme, Cambridge, MA) by adding 100 ng of capture mAb (in 0.05 ml volume). The plates were then washed with PBS/Tween and incubated with 25% FCS (Sigma) in PBS/ Tween. The plates were washed again, and serial dilutions of supernatant medium were added to the wells and incubated for 4 h. After repeated washes, the concentration of IL-7 was measured using biotinylated polyclonal antibodies to murine IL-7 (R & D Systems, Minneapolis, MN), streptavidin–peroxidase and o-phenylenediamine chloride as a substrate (Sigma). A standard curve using recombinant murine IL-7 (Genzyme) followed a linear pattern over a concentration range of 0.15–10 ng/ml. The lower level of detectability of IL-7 was 100 pg/ml.

**bcl-xL expression by RT-PCR**

RNA was extracted from sorted pre-B cells using TRI Reagent (Sigma) according to the manufacturer’s instructions. cDNA was synthesized by reverse transcription using the the 1st Strand cDNA Synthesis kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. PCR was used to amplify a 209 bp fragment of $bcl-x_L$ and control GAPDH mRNA using PCR Master Kit (Boehringer Mannheim) and a thermal cycler (Perkin-Elmer, Branchburg, NJ), and the
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Fig. 2. TUNEL assay of cultured pre-B cells from a 2- and 18-month-old mice. Bone marrow cells were depleted of slg⁺ and CD43⁺ cells, cultured 3 days, depleted again of CD43⁺ cells, permeabilized, and stained with B220–Cy-Chrome (FL3) and slg–PE (FL2) and labeled dUTP (FL1). During analysis, intact cells were gated for B220⁺ (FL3⁺) cells. The lower panel displays the CD43 expression after depletion with anti-CD43 antibody. The number in each square represents the percentage of each subset.

Intracellular Bcl-xL
Aliquots of sorted pre-B cells, 0.2×10⁶ were fixed in the mixture of 95% methanol and 5% glacial acetic acid solution (17), washed, resuspended in 10 mM Tris, 150 mM NaCl, 0.02% Tween 20 solution, incubated with polyclonal rabbit anti-BCL-X antibody (PharMingen) or rabbit Ig as a negative control and anti-rabbit Ig–FITC (Sigma), and analyzed by flow cytometry.

Detection of DNA fragmentation in pre-B cells
Aliquots of 10⁶ slg⁺CD43⁺ bone marrow cells were selected by flow cytometry and cultured with adherent cells (10⁶/ml/well) from the same animal. Non-adherent cells were then collected, washed in PBS, depleted of CD43⁺ cells again, stained with Cy-Chrome-labeled anti-mouse B220 and PE-labeled anti-mouse IgM/IgG/IgA heavy and light chain-specific antibody, and fixed in 4% paraformaldehyde (Sigma). After repeated washes, cells were stored overnight in 70% ethanol. Prior to the analysis, the cells were washed in PBS and DNA breaks were assessed by the TdT-
mediated incorporation of digoxigenin-11-dUTP using the ApopTag In Situ Apoptosis Detection kit (Oncor, Gaithersburg, MD) according to the manufacturer’s instructions. Cells not exposed to TdT were used as negative controls. Incorporated digoxigenin-11-dUTP was revealed with FITC-labeled antibody to digoxigenin (Oncor). The samples were analyzed by flow cytometry as described.

Annexin V binding by sorted pre-B cells
Aliquots of $0.1 \times 10^6$ freshly isolated pre-B cells were incubated with biotinylated annexin V (Trevigen, Gaithersburg, MA) according to the manufacturer’s instructions followed by the incubation with streptavidin–PE and flow cytometry analysis using FACScan. Pre-B cells incubated with streptavidin–PE only were used as negative controls. Specific and non-specific binding was repeatedly tested using cultured apoptotic cells.

Statistical analysis
Results are expressed as the mean ± SD. The numbers of pre-B cells and IL-7 expression have been analyzed using Student’s t-test, while the data from sorted subsets have been analyzed using the $\chi^2$ test. Results were considered significant at $P < 0.05$.

Results
Decreased number of pre-B cells in the bone marrow of 18-month-old mice
The average number of bone marrow pre-B cells with a phenotype of $\text{B220}^+\text{CD43}^-\text{sIg}^-$ in the bone marrow from 18-month-old mice was significantly lower than in 2-month-old mice (Table 1 and Fig. 1). In contrast, the number of pro-B cells expressing $\text{B220}^+\text{CD43}^+\text{sIg}^-$ was comparable in 2- and 18-month-old mice (Table 1).

Comparable secretion of IL-7 by bone marrow cells from 2- and 18-month-old mice
Adherent bone marrow cells from 2- and 18-month-old mice in short-term culture secreted comparable amounts of IL-7 (Table 2). Non-adherent bone marrow cells did not secrete detectable IL-7 (data not shown).

Decreased survival of bone marrow pre-B cells from 18-month-old mice
To study the survival of bone marrow B cell precursors from 2- and 18-month-old mice, we have cultured purified populations of pro-B or pre-B cells with S17 stromal cells (Table 3). Cultures of pro-B cells from both old and young mice after 4 days had pre-B cells but not pro-B cells. However, the number of pre-B cells was significantly ($P < 0.01$) lower in pro-B cell bone marrow cultures from old as compared to young mice. Similarly, in 4 day cultures of pre-B cells, there were significantly fewer pre-B and $\text{sIg}^+$ cells detectable in cultures from old than young mice.

Higher percentage of apoptotic cells in bone marrow pre-B cells from 18-month-old mice
The decreased survival of bone marrow pre-B cells in cultures from old compared to young mice suggested the possibility that the rate of apoptosis of bone marrow pre-B cells is higher in old mice. To test this hypothesis, the number of apoptotic pre-B cells from young and old mice were determined after being cultured with adherent cells for 3 days. There was a significantly higher percentage of apoptotic cells ($P < 0.01$) in cultures from old compared
bcl-xL mRNA and protein are decreased in pre-B cells from 18-month-old mice

Programmed cell death during lymphoid cell development can be prevented by Bcl-2, Bcl-xL, and related gene products; bcl-xL is known to be preferentially expressed by pre-B cells (18). We studied bcl-xL expression in sorted pre-B cells from 2- and 18-month-old mice, and found that both mRNA and protein levels are markedly decreased in CD43^B220^sIg^- bone marrow cells from 18-month-old mice (Fig. 4). Quantitat-
ive estimates of these data suggest that the expression of bcl-x\(_L\) by bone marrow pre-B cells from 18-month-old mice is 10% or less of that expressed by pre-B cells from 2-month-old mice.

**Discussion**

Recent studies have demonstrated that there is a decreased number of bone marrow pre-B cells in mice >4 months old and this number continues to decline throughout life (14). As the development of early B cell precursors depends on IL-7, it was reasonable to ascribe the age-associated decline in the number of pre-B cells to a decreased production of or response to IL-7 in the bone marrow. This hypothesis is supported by the finding that the lower number of pre-B cells in 24-month-old mice is associated with a reduced response of their pro-B cells to IL-7 (15).

We found no evidence that IL-7 production by adherent bone marrow cells was different in 2- and 18-month-old mice. As the effect of IL-7 is determined not only by its secretion, *per se*, but also by its binding and the cascade of events leading to the cellular response (19–29), survival of bone marrow B cell precursors in the presence of IL-7 produced by stromal cells line has been tested. Pro-B cells cultured with the S17 stromal cell line, which produces IL-7, change their phenotype to that typical of pre-B cells. Pro-B cell from old mice generate fewer pre-B cells in such cultures than do pro-B cells from young mice. These data are consistent with those previously reported (15).

As we could not detect any differences in factors, such as IL-7, that might impair the generation of pre-B cells in 18-month-old mice, we investigated whether the percentage of pre-B cells undergoing programmed death is higher in old than in young mice. We cultured bone marrow pre-B cells from 18- and 2-month-old mice, and found that there was a significantly higher percentage of pre-B cells with DNA strand breaks, an early marker of apoptosis, in cultures from the 18-month-old mice as compared to 2-month-old mice. We also demonstrated an age-associated increase in the percentage of apoptotic pre-B cells *ex vivo* using the more sensitive annexin V binding assay.

The consequences of increased apoptosis of pre-B cells in old mice are not clear. One possible consequence is a decreased production or diversity of B cells generated in the bone marrow of old mice. Although it has been reported that the output of newly generated B cells is relatively constant throughout life (14), there is clear evidence that the diversity of the B cell repertoire, with respect to affinity, isotype and idiotype, decreases with age (30). Another factor which contributes to the decreased diversity of the B cell repertoire with age is clonal expansions of B cells which have been detected in virtually all mice 18 months of age and older (31).

Finally, we attempted to define a possible mechanism underlying the increased percentage of pre-B cells undergoing programmed cell death in 18-month-old mice. As Bcl-2 and Bcl-x\(_L\) are known to block apoptosis in lymphoid cells and because bcl-x\(_L\) is expressed in pre-B cells (18), we focused on this gene. We found a significant decrease in bcl-x\(_L\) mRNA and protein expressed by bone marrow pre-B cells from 18-month-old mice compared to 2-month-old mice. In summary, we conclude that the age-associated decrease in the number of bone marrow pre-B cells is due to an increased rate of apoptosis of these cells associated with their decreased expression of the anti-apoptotic gene bcl-x\(_L\).

**Acknowledgements**

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**Abbreviations**

PE phycocerythin

**References**

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