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Proliferation of Bone Marrow Pro-B Cells Is Dependent on Stimulation by the Pituitary/Thyroid Axis

Melanie P. Foster, Encarnacion Montecino-Rodriguez, and Kenneth Dorshkind

The frequency and absolute number of pro-B, pre-B, and B cells in the bone marrow of the hypothyroid strain of mice are significantly reduced compared with those of their normal littermates. To investigate why this is the case, various B cell developmental processes were examined in the thyroid hormone-deficient mice. These studies revealed that the frequency of pro-B cells in the S-G2/M phase of the cell cycle was significantly reduced in hypothyroid mice. That thyroid hormone deficiency was responsible for this proliferation defect was established by demonstrating that treatment of hypothryoid mice with thyroxine resulted in a specific increase in the frequency and total number of cycling pro-B cells. The latter effect was paralleled by increases in the frequency and number of bone marrow B lineage cells. Additional in vitro experiments revealed that at least some thyroid hormone effects were directly mediated on the bone marrow. Taken together, these data demonstrate that thyroid hormones are required for normal B cell production in the bone marrow through regulation of pro-B cell proliferation and establish a role for the pituitary/thyroid axis in B cell development. The Journal of Immunology, 1999, 163: 5883–5890.

B lymphocytes, like all hematopoietic cells, arise from pluripotent hematopoietic stem cells (1, 2) within the medullary cavity of the bone marrow (3, 4). As committed B cell precursors differentiate, they express various cell surface determinants (5, 6) and rearrange their Ig heavy and light chain genes (7, 8). Although the phenotypic stages of B lymphopoiesis are increasingly well defined, the extracellular signals regulating the production of B lineage cells remain incompletely characterized. Stimuli derived from the bone marrow microenvironment, such as direct cell contact with stromal cells and secreted growth factors, are one source of these signals (3, 4, 9, 10). For example, stromal cell-derived IL-7 (11) acts as a pro-B cell proliferation and differentiation signal (12), and additional stromal cell-derived factors have been shown to synergize with IL-7 to stimulate proliferation of B cell progenitors (10).

It is becoming increasingly apparent that signals of extramedullary origin also affect normal B cell development, and hormones derived from various endocrine tissues are important in this regard (13). For example, B cell development in pregnant mice is suppressed by elevated estrogen levels (14). Conversely, other studies have revealed that hormones produced by the thyroid gland act as obligate, positive regulators of primary B lymphopoiesis (15, 16). This latter conclusion is based on the examination of B lymphopoiesis in several strains of mice deficient in the production of one or more anterior pituitary-derived hormones due to naturally occurring or induced genetic defects (16, 17). These studies revealed that, whereas B lymphopoiesis is normal in animals with defective production of growth hormone, insulin-like growth factor-I, or prolactin, the frequency and absolute number of B lineage cells is significantly decreased only in thyroid hormone-deficient strains. For example, hypothyroid (hyt/hyt) mice produce ~10% of normal thyroid hormone levels due to the inability of thyroid epithelial cells to bind thyroid-stimulating hormone (18–20). The frequency of B lineage cells was reduced by up to 70% in hyt/hyt mice, and this deficiency could be completely corrected by administration of thyroxine (T4) (16). These hematopoietic defects in thyroid hormone-deficient mice only affect cell production in the B lineage, given that no significant differences in myeloid cell frequencies or numbers were observed (16).

The mechanism(s) responsible for the specific decrease in the production of B lineage cells in hyt/hyt mice has not been defined. Thyroid hormones have been shown to regulate cell proliferation, differentiation, and death during embryogenesis and postnatal life (reviewed in Ref. 21), and normal B cell development is dependent on the coordination of all of these processes. For example, the most immature B cell precursors, referred to as pro-B cells, proliferate extensively in the bone marrow to maintain the size of the pro-B cell pool and to generate progeny that will differentiate into Ig-expressing cells (6, 22, 23). The latter event is dependent on the successful recombination of genes that encode the Ig molecule, and those B lineage precursors that are not successful at doing so undergo apoptosis and are eliminated from the bone marrow. Thus, the ultimate production of a B lymphocyte is a dynamic process involving cell growth, cell maturation, and cell death (5, 8, 24–26), and the tempo of any one of these events could be negatively impacted by the absence of thyroid hormones.

To determine how thyroid hormones affect B lymphopoiesis, various B cell developmental processes were examined in hyt/hyt mice. The results of these analyses indicate that the frequency of proliferating pro-B cells is reduced in that strain and that treatment of the mice with thyroxine increased both the frequency and total number of cycling pro-B cells. Taken together with the results of additional in vitro assays demonstrating that the number of B lineage cells is increased in the presence of thyroid hormones, these data suggest that products of the pituitary/thyroid axis regulate the proliferative potential of developing B cell precursors.
Materials and Methods

Mice

BALB/c BJ (BALB/c), C.RF-hythy/hyt, and C.RF-hyt/hyt (which are either hyt/+ or +/+ mice) were obtained from The Jackson Laboratory (Bar Harbor, ME). The hythy/hyt mice were 8 to 12 wk of age and had been removed from normal litters for at least 2 wk before the experiment to eliminate potential hormonal effects transferred to them. All of the mice were housed in microisolator cages under laminar flow conditions in the Division of Laboratory Animal Medicine vivarium at UCLA.

In some experiments, mice received an i.v. injection of 150 mg/kg 5-fluorouracil (5-FU, Sigma, St. Louis, MO (2, 27)) 8 days before analysis. In addition, some of the 5-FU-treated animals received a daily s.c. injection of 2 μg of rat-thyroxine (T4; T-0881; Sigma) or saline for 8 days. T4 was used for these studies because it is more stable and has a longer duration of in vivo activity than triiodothyronine (T3) (28).

Preparation of cell suspensions

Mice were killed by cervical dislocation. A single-cell suspension of bone marrow cells was obtained by flushing femurs and tibiae with 3 ml nMEM (Life Technologies, Grand Island, NY). Cells were counted in a hemacytometer, and cell viability, determined by eosin dye exclusion, always exceeded 95%.

Immunofluorescence

Bone marrow cells were incubated with the following Abs: FITC-conjugated anti-IgM (Southern Biotechnology, Birmingham, AL), FITC-conjugated heat-stable Ag (HSA), PE-conjugated anti-CD45R (B220, clone RA3-6B2), biotin-conjugated anti-CD43 (clone S7), or FITC-conjugated anti-CD25 (clone 7D4; Pharmingen, San Diego, CA). Biotinylated Abs were revealed with FITC-conjugated streptavidin (Southern Biotechnologoy). After RBC lysis by osmotic shock, samples were incubated with an anti-murine FcyII and III receptor Ab (CD16/32, clone 2.4G2, Pharmingen) to reduce nonspecific labeling before the addition of one or more of the above Abs. All stainings were conducted in Ca++-,- Mg++-free PBS (Life Technologies) on ice. After the last wash, 10^5 cells from each sample were analyzed on a FACSscan (Becton Dickinson, San Jose, CA) with CELL Quest software (Becton Dickinson).

Enrichment of CD45R+ cells

Bone marrow CD45R+ cells were enriched by using a magnetic activated cell sortor column (Miltenyi Biotec, Auburn, CA) according to the manufac- turer’s protocol. Briefly, after incubation with anti-CD32/CD16 Ab, cells were incubated with anti-murine CD45R-conjugated superparamagnetic beads in Ca++-, Mg++-free PBS, 5 mM EDTA, 0.5% BSA, pH 7.2 (all from Sigma) for 25 min at 6–8°C under constant agitation. The cells were then washed and loaded on the magnetic activated cell sortor column placed in its magnetic support. The column was rinsed with 3 to 4 times the column volume to remove the negative cells, and the positive fraction was eluted from the column after its removal from the magnetic support. CD45R+ cell enrichment ranged from 60 to 95% depending on the experiment.

In some experiments, CD45R+ slgM+ cells were purified on a FACStar plus cell sorter (Becton Dickinson) after incubations with FITC-anti-IgM and PE-anti-CD45R Abs. Incubation with anti-murine CD45R-conjugated superparamagnetic beads or fluorochromes did not interfere with subsequent immunofluorescence analysis or in vitro culture experiments (data not shown).

Cell cycle analysis

Cells were fixed in 0.5% paraformaldehyde for 15 min at room temperature. The cells were then permeabilized with 70% ethanol and resuspended in Ca++-, Mg++-free PBS supplemented with 1 μg/ml 7-amino actinomy- cin D (7-AAD) (Calbiochem, San Diego, CA). At least 3 × 10^4 cells were acquired with a FACSscan, and their cycling status was estimated using Modfit LT software (Verity Software House, Topsham, ME). The coeffi- cient of variation for the G0/G1 peak obtained during these analyses was on average <5%.

Detection of apoptotic cells

Apoptotic cells were detected by their increased permeability to the DNA dye 7-AAD (29). After immunostaining for CD45R, CD43, and/or IgM expression, 5 × 10^5 CD45R+ or cultured bone marrow cells were incubated with 1 μg/ml 7-AAD in Ca++, Mg++ free PBS. Following 15–30 min incubation, cells were analyzed on a FACScan equipped with a double discrimination module. Gates were set on the basis of staining with sec- ondary reagent alone. Dead cells were excluded based on their forward and side scatter profile. Apoptosis was determined with CELL Quest software (Becton Dickinson).

Apoptotic cells were also detected by annexin V staining (R&D Sys- tems, Minneapolis, MN). Following immunostaining for CD45R expres- sion, 3 × 10^6 cells were resuspended in binding buffer (10 mM HEPES/ NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2) and incubated with FITC-conjugated annexin V for 15 min at room temperature in the dark according to the manufacturer’s instructions.

Long term bone marrow cultures

Long term myeloid cultures were established as described (30). After 4 wk, these cultures were switched to B lymphoid-permissive conditions (31, 32). At the time of switch and at each weekly feeding thereafter, the culture medium was supplemented with 10^-8 M T3 or saline. T3 was used in these studies because it was not clear that all the requirements for conversion of T3 to T4, the most active form of the hormone (28), would be present in these cultures. Cells harvested from at least three cultures per condition were analyzed weekly for emergence of CD45R+ slgM+ and slgM- cells by immunofluorescence.

RT-PCR and Southern blot analysis

Subpopulations of bone marrow B lineage cells from BALB/c mice were purified into fraction A (HSA+, CD45R-, CD43+), fraction B + C (HSA-, CD45R+, CD43+), fraction D (CD45R-, CD43+, slgM-), and fraction E (SLAM+, B220+, CD43+, slgM+) (10). The cells were obtained according to the manufacturer’s instructions. Formalin-fixed, paraffin-embedded tissues were sectioned (6-μm thick) and hybridized with a T3 probe. The hybridization signal was detected with the CDP-star detection system (Pharmacia Biotech, Piscataway, NJ) followed by autoradiography. The radioactivity was quantitated with a Phosphorlmager (Molecular Dynamics, Sunnyvale, CA). For some experiments, cells were stained with FITC-conjugated annexin V (R&D Systems) or PI (Sigma) to detect early and late apoptotic cells, respectively.

RT-PCR was performed in a first order of each primer, thyroxine hormone receptor (TR) α gene common 5'-primer, CCAAGCTTTACGGACACAGC (TRα1); 3'-ACGACATTCTCGGTATCCAG (549 bp). TRβ2, 3'-AGACCTCGACAGCGAGCTGTC (933 bp). TRβ gene common 3'-primer, ACAGACCTTACCTTTGTCTAAGTAG (33); TRβ1, 5'-GGGTGTCAACAGGTTCACACAGT (410 bp). TRβ2, 5'-GGTATTTACCCITCTTGTGTGTA GTGAT (480 bp). GAPDH primers 5'-CCATGGAGAAGCTGGGG and 3'-CAAAAGTGGTAGTGTCGCC (200 bp) were used to ensure DNA integrity.

Proliferation assay

Sorted CD45R+ slgM- cells (10^3/well) were distributed in 96-well plates, either over irradiated confluent S17 stromal cells (35) or without stroma, in Iscove’s Medium (Life Technologies) supplemented with 5% FBS and l-glutamine (Life Technologies) and incubated for 3 days in a humidified incubator at 37°C and 5% CO2/air. Some wells were additionally supplemented with 10^-8 M T3 and/or 50 U/ml recombinant murine IL-7 (Bio- source International, Camarillo, CA). A parallel set of cultures was also established with PBS from which 85% of the T3 and 92% of T4 had been depleted (36), as confirmed by RIA performed in the UCLA Department of Endocrinology. All cultures were pulsed with 1 μCi/well [methyl-3H]thymidine during the last 16 h of cul- ture, and specific incorporation by the cells was determined.

Statistics

Data were analyzed using a single-tailed Student t test.
Results

B cell development is suppressed in thyroid hormone-deficient mice

The data in Fig. 1 show, in agreement with previously published results (16), that the frequency of CD45R+ IgM+ pro/pre-B and surface IgM+ B cells is lower in hyt/hyt mice than in their normal +/+ littermates. The absolute number of B lineage cells is also lower in the hormone-deficient mice (Ref. 16 and data not shown).

B lineage cells in hyt/hyt mice do not have an increased rate of apoptosis

An increased rate of apoptosis could explain the decreased number and frequency of B lineage cells in hyt/hyt mice. To investigate this possibility, the frequency of apoptotic hyt/hyt and +/+ bone marrow B lineage cells was determined by measuring membrane permeability to 7-AAD. Attempts were made in initial experiments to assess the frequency of apoptosis in B lineage cells in unfractonated hyt/hyt bone marrow, but the low numbers of pro-B/pre-B cells in that strain made this analysis difficult. Therefore, CD45R+ cells from the bone marrow of hyt/hyt mice were enriched on a magnetic column before analysis in subsequent experiments.

Large CD45R+ slgM+ cells are the B lineage population with the highest incidence of apoptosis (37). Because cells with these physical and phenotypic characteristics are included in the CD45R+ CD43+ pro-B cell population (38), initial experiments focused on defining the frequency of apoptotic cells in that compartment. As shown in Table I, the frequency of apoptotic CD45R+ CD43+ pro-B cells in hyt/hyt and +/+ mice was comparable. A similar conclusion was reached when the analysis was performed on slgM+ B cells, which have the next highest incidence of apoptosis (37), and CD45R+ slgM+ cells or when the same analyses were repeated using propidium iodide staining to detect hypodiploid DNA (data not shown).

Because apoptotic cells are rapidly deleted from the bone marrow, the number detectable at any one time is low (37). Therefore, a short term culture system described by Lu and Osmond (37) that allows the accumulation and enumeration of apoptotic cells was used. Bone marrow cells from both hyt/hyt and +/+ mice were incubated for up to 6 h, and apoptosis was examined by both membrane permeability and annexin V staining. The frequency of apoptotic B lineage cells from both hyt/hyt and +/+ mice was similar in this assay (data not shown).

Bone marrow B lineage cells from hyt/hyt mice show a reduced proliferative capacity

To determine whether thyroid hormone deficiency affects the proliferative status of B lineage cells in hyt/hyt mice, their cycling status was examined. Similar to what was encountered in the apoptosis studies, attempts to assess the frequency of S-G2/M phase B lineage cells in unfractonated hyt/hyt bone marrow were unsuccessful due to the low numbers of pro-B/pre-B cells (data not shown). Because most cycling B lineage cells are found in the pro-B cell compartment (6, 22), in subsequent studies hyt/hyt and +/+ bone marrow cells were labeled with a combination of Abs that would permit the cycling status of enriched CD45R+ CD43+ pro-B cells to be assessed.

The data in Table II show results from three experiments in which the cell cycle status of these enriched CD45R+ CD43+ pro-B cells from hyt/hyt mice and their +/+ littermates was compared. A consistent finding in all three experiments was that the frequency of pro-B cells in the S-G2/M phase of the cell cycle was significantly lower in hyt/hyt mice than in their +/+ littermates. In agreement with Hardy et al. (6), the frequency of S-G2/M phase CD45R+ CD43+ pro-B and B cells was comparatively lower, and no significant differences were observed in the cycling status of these populations in hyt/hyt and +/+ mice. For example, in one representative study, 5.9% of hyt/hyt and 4.8% of +/+ CD45R+ CD43+ cells were in the S-G2/M phase of the cell cycle.

The CD25 cell surface determinant can be used to identify cells at the late pro-B (fraction C’) to pre-B cell (fraction D) stages of development (39). To define further the pro-B cell stage at which the proliferation defect is manifest, additional studies examined CD45R+ CD25+ bone marrow cells from both hyt/hyt mice and their +/+ littermates. The data demonstrate that there was no difference in the frequency of proliferating large or small CD45R+ CD25+ bone marrow cells between hyt/hyt mice and their +/+ littermates (data not shown).

Bone marrow pro-B cells from 5-FU-treated hyt/hyt mice show a reduced proliferative capacity

The above results strongly suggest that the proliferative potential of pro-B cells is compromised in hyt/hyt mice. To accentuate this defect, de novo pro-B cell proliferation was induced by treating the mice with the cytostatic drug 5-FU. After treatment, cycling bone marrow cells are eliminated (27, 40), and resting immature hematopoietic progenitors are driven into cell cycle to replenish the myeloid and lymphoid compartments in the marrow. Whether or not hyt/hyt mice would also show a pro-B cell proliferation defect in this model was thus investigated.

Table I. Pro-B cells in the bone marrow of hyt/hyt mice do not have an increased rate of apoptosisa

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Apoptotic CD45R+CD43+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>+/+</td>
<td>8.5</td>
</tr>
<tr>
<td>hyt/hyt</td>
<td>12.3</td>
</tr>
</tbody>
</table>

a Bone marrow cells were pooled from 2–4 mice for each experiment, and CD45R+ CD43+ cells were enriched by magnetic column.

Table II. Frequency of cycling pro-B cells in the bone marrow of hyt/hyt mice is reduced

<table>
<thead>
<tr>
<th>Strain</th>
<th>Frequency of Cells in S-G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>+/+</td>
<td>22.1</td>
</tr>
<tr>
<td>hyt/hyt</td>
<td>14.5</td>
</tr>
</tbody>
</table>

b Bone marrow cells were pooled from 2–4 mice for each experiment. Data were analyzed using Modfit LT software.

c Significantly different from +/+ controls at p < 0.025.
Effect of 5-FU on the frequency and cell cycle status of pro-B cells in the bone marrow of hyt/hyt and +/− mice. FACScan profiles of B lineage cells and cell cycle histograms of pro-B cells in the bone marrow of +/− (A) and hyt/hyt (B) mice after treatment with 5-FU. Data are representative of one of three experiments using a total of 2−4 mice per group and were analyzed using ModFit LT software.

Fig. 2 shows a representative experiment demonstrating that the frequency of cycling CD45R+CD43+ pro-B cells in 5-FU-treated hyt/hyt mice (84.5%) was significantly lower than in their +/− littermates (96.2%) 8 days after 5-FU treatment. The latter time point was chosen based on a report showing that at 8 days post-5-FU treatment, immature hemopoietic progenitors are actively cycling (41), and preliminary experiments in which the cell cycle status of bone marrow from 5-FU-treated mice was examined at different times after drug injection (data not shown). Similar results were also obtained in two additional experiments (Table III).

The data in Table III further demonstrate that the absolute number of cycling pro-B cells in hyt/hyt mice was lower than in their +/− littermates and that, consistent with previous findings (16), hyt/hyt mice had a significantly lower frequency and a reduced absolute number of CD45R+CD43+ pro-B cells.

Thyroid hormones have direct effects on the bone marrow

To examine whether thyroid hormones can directly affect the bone marrow, the biologically active form of thyroid hormone, T3, was added to myeloid long term bone marrow cultures at the time of their transfer to B lymphoid-permissive conditions (32). In this system, myelopoiesis declines and B lymphopoiesis initiates and plateaus at 4 wk after transfer to the lymphoid culture conditions. As shown in Fig. 4, the kinetics of when B lineage cells emerge in the cultures was not affected by the addition of T3 to the cultures. However, between weeks 1 and 3, the period during which the transition from myelopoiesis to B lymphopoiesis occurs, the frequency and absolute number of CD45R−sIgM− cells were significantly increased in the T3-supplemented cultures (Fig. 4 and Table V).

TRs are expressed throughout B cell development

In view of the ability of thyroid hormones to affect bone marrow B lymphopoiesis, the expression of TRs was examined on subpopulations of bone marrow B lineage cells by RT-PCR and Southern blot analysis. TRs are expressed from two genes, α and β, and both produce different isoforms due to alternative splicing

### Table III. Effect of 5-FU on the frequency and cell cycle status of pro-B cells in the bone marrow of hyt/hyt and +/− mice

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+/−</td>
<td>25.90</td>
<td>1.60</td>
<td>96.20</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>hyt/hyt</td>
<td>18.40</td>
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<tr>
<td>2</td>
<td>+/−</td>
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<tr>
<td></td>
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<tr>
<td>3</td>
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<td>59.40</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>hyt/hyt</td>
<td>10.60</td>
<td>0.35</td>
<td>50.60</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* Bone marrow cells were pooled from 3−4 mice in each experimental group. Data from 3 × 10⁶ cells were analyzed using ModFit LT software.
* hyt/hyt mice are significantly different from +/− controls at p < 0.025.
* Absolute numbers (Ab. No.) were determined by multiplying the percentage of CD45R+CD43+ cells by the total number of bone marrow cells per mouse.
* hyt/hyt mice are significantly different from +/− controls at p < 0.005.
* Ab. No. were determined by multiplying the absolute number of CD45R+CD43+ cells by the percentage of cells in S-G2/M. 
events (42–44). As shown in Fig. 5, TRα1 and TRβ1 were constitutively expressed throughout B cell development, whereas TRβ2 was not detected. On the other hand, TRα2 was detected in fractions A–C, down-regulated in fraction D, and up-regulated in fraction E + F.

Thyroid hormones do not function as pro-B cell mitogens or proliferation cofactors

The expression of the TR on bone marrow pro-B cells provided the rationale for assessing whether or not thyroid hormones had direct effects on cells in that compartment. Purified CD45R⁺sIgM⁻ bone marrow cells from hyt/hyt mice were cultured with 10⁻⁸ M T3 for 72 h. Because hyt/hyt cells were “maintained” in a thyroid hormone-deficient environment, it was reasoned that hormone effects would be more demonstrable on these targets than on similar cell populations from normal animals. As shown in Fig. 5, T3 did not stimulate proliferation of CD45R⁺sIgM⁻ cells in medium containing FBS or thyroid hormone-depleted FBS in cultures analyzed at 3 and 7 days (data not shown). However, cells cultured in either of these conditions exhibited vigorous proliferation in response to the pro-B cell growth factor IL-7. No proliferative effects of T3 were noted when cultures were examined at 24 and 48 h (data not shown).

A class of proliferation cofactors, that includes flk2/flt3 ligand and c-kit ligand, has been described (10). Cytokines in this category do not act alone to stimulate pro/pre-B cell proliferation but can synergize with IL-7. To determine whether thyroid hormones function in this manner, the proliferative response of CD45R⁺sIgM⁻ cells to IL-7 and IL-7 + thyroid hormones was compared. The data in Fig. 6 further show that 3 days after initiation of cultures, the proliferative response of cells under these two combinations was comparable. A similar conclusion was reached when in repeat experiments the culture time was extended to 7 days (data not shown).

Finally, additional experiments were performed in which sorted CD45R⁺sIgM⁻ cells from hyt/hyt mice were seeded over irradiated S17 stromal cells in the presence of IL-7 alone, T3 alone, or IL-7 + T3 for 1–3 days. T3 again had no effect on cell proliferation under these conditions (data not shown).

Table IV. Effect of T4 on the cycling status of pro-B cells in 5-FU-treated hyt/hyt mice

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Treatment</th>
<th>%b (Ab. No.) × 10⁴⁺</th>
<th>% S-G2/M</th>
<th>(Ab. No. S-G2/M) × 10⁴⁺d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Saline</td>
<td>6.90</td>
<td>0.16</td>
<td>70.80</td>
<td>0.11</td>
</tr>
<tr>
<td>T4</td>
<td>19.60</td>
<td>0.54</td>
<td>81.00</td>
<td>0.40</td>
</tr>
<tr>
<td>2. Saline</td>
<td>10.60</td>
<td>0.35</td>
<td>50.60</td>
<td>0.18</td>
</tr>
<tr>
<td>T4</td>
<td>23.17</td>
<td>1.10</td>
<td>55.50</td>
<td>0.60</td>
</tr>
</tbody>
</table>

*a Bone marrow cells were pooled from 3–4 mice in each experimental group. Data from 3 × 10⁴ cells were analyzed using Modfit LT software.

*b Absolute numbers (Ab. No.) were determined by multiplying the percentage of CD45R⁺CD3⁺ cells by the total number of bone marrow cells per mouse.

c Absolute numbers (Ab. No.) were determined by multiplying the absolute number of CD45R⁺CD3⁺ cells by the percentage of cells in S-G2/M.

Discussion

B lymphocyte development in the bone marrow is regulated by a variety of extracellular signals that affect the differentiation, survival, and proliferation of developing B lineage cells (3–5, 9). Although many of these stimuli are produced by the bone marrow microenvironment, there is growing evidence that factors of extramedullary origin also affect primary B lymphopoiesis. In this

![FIGURE 3](image-url)  
**FIGURE 3.** Cell cycle status of bone marrow B lineage cells from hyt/hyt mice after T4 treatment. FACScan profiles of B lineage cells and cell cycle histograms of pro-B cells in the bone marrow of hyt/hyt mice that received a daily injection of either saline (A) or T4 (B) after 5-FU treatment. Data are representative of two identical studies using a total of 3–4 mice per group and were analyzed using ModFit LT software.

![FIGURE 4](image-url)  
**FIGURE 4.** Effects of T3 on B lymphopoiesis in long term bone marrow cultures. The frequency of CD45R⁺sIgM⁻ (■) and CD45R⁺sIgM⁺ (○) cells in cultures supplemented with 10⁻⁸ M T3 (●, □) or medium only (○, □) is shown. *p < 0.001, and **p < 0.0025, indicates a significant difference between cultures with and without T3. Each time point is based on analyzing 3 to 5 cultures. Results are representative of three experiments.
regard, recent reports from this laboratory in which mice with defects of the pituitary/thyroid axis were studied revealed that thyroid hormones are obligate, positive regulators of B cell differentiation (16). For example, the absolute number and frequency of B lineage cells are reduced in hyt/hyt mice compared with their normal littermate controls. However, although the initial evaluation of this strain clearly documented a role for thyroid hormones in B cell development (16), how they mediate their effect(s) was not determined. Accordingly, the aim of the present study was to determine how thyroid hormone affect B lymphopoiesis.

Reduced concentrations of thyroid hormones in hyt/hyt mice could compromise B lymphopoiesis through effects on the differentiation, survival, and/or proliferation of B lineage cells. Although the actions of thyroid hormones on the differentiation of B cell progenitors cannot be excluded, such an effect seems unlikely because all stages of B lymphopoiesis are present in hyt/hyt mice. Furthermore, the frequency of the most immature, committed CD45R⁺CD43⁻/⁺CD45R−CD43⁺HSA⁻ B cell precursors (Hardy fraction A) is comparable in hyt/hyt mice and their +/+ littersmates (16), suggesting that the differentiation of pluripotent stem cells and/or lymphoid stem cells into the B cell lineage is not compromised. Therefore, it was logical to propose that the deficiency of B lineage cells in the absence of thyroid hormone was caused by a decrease in survival or proliferation of committed B cell precursors. No evidence to support a role for thyroid hormones in the survival of B lineage cells was obtained in this study. Even though multiple techniques allowing detection of cells at early and late stages of apoptosis were used, no differences in the frequency of apoptotic B lineage cells between hyt/hyt mice and their normal littermates were revealed. Therefore, subsequent experiments focused on whether or not thyroid hormones could affect the proliferation of B cell progenitors.

In the first set of experiments, the cell cycle status of CD45R⁺CD43⁻ pro-B cells in hyt/hyt mice was compared with that in their +/+ littermates. The pro-B cell compartment was the focus of these studies because B cell progenitors with the highest rate of proliferation are included in it (6), and no differences in the frequency of cycling CD45R⁺CD43⁻ (pre-B and B) cells in hyt/hyt and +/+ mice were observed. The data demonstrated a requirement for thyroid hormones in pro-B cell proliferation. On average, 23.8% of CD45R⁺CD43⁻ cells in hyt/hyt mice were in cycle, whereas in the +/+ littersmates 29% were in the S-G 2 /M phase of the cell cycle. The expression of the CD25 cell surface determinant was used to delineate at which pro-B cell stage the proliferative defect in hyt/hyt mice occurred. The results showed that there was no difference in the frequency of proliferating CD25⁺ pro-B cells between hyt/hyt mice and their normal littersmates, suggesting that fractions A, B, and early C were affected by the absence of thyroid hormone. The pro-B cell compartment is estimated to contain $\sim 1$ million cells (45) that are capable of both differentiation into pre-B cells and self-renewal and that, together, maintain the pro B-cells pool at a constant size. The decreased proliferation of pro-B cells in hyt/hyt mice apparently has profound consequences over time, as substantiated by the significantly lower frequency and absolute numbers of cells in that compartment and on the size of more mature, downstream populations.

This working hypothesis is consistent with the results of Arpin and colleagues who have shown that mice with a targeted disruption of the α TR also have a defect in pro/pre-B cell proliferation (C. Arpin and J. Marvel, unpublished observations). Thus, whereas the data herein document a pro-B cell proliferation defect in animals with deficient production of thyroid hormones (the ligand), a similar deficiency has been observed in mice with aberrant receptor expression.

To make the requirement for thyroid hormones in pro-B cell proliferation more evident, subsequent experiments in which mice were treated with 5-FU were conducted. In general, the frequency of CD45R⁺CD43⁻ bone marrow pro-B cells in the 5-FU-treated hyt/hyt and +/+ mice was markedly higher than in their respective saline treated controls or in unmanipulated mice. This result is likely due to the fact that few mature cells are present in the marrow 8 days after 5-FU treatment; there is thus a relative increase in the frequency, and possibly the absolute number, of the pro-B

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**Table V. Effect of T3 on production of CD45R⁺ sIgM⁻ cells in long term bone marrow cultures**

<table>
<thead>
<tr>
<th>Wk Under Lymphoid Conditions</th>
<th>Absolute No. CD45R⁺ sIgM⁻ Cells⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1 (0.01–0.2)</td>
</tr>
<tr>
<td>1</td>
<td>1.6 (0.7–2.8)</td>
</tr>
<tr>
<td>2</td>
<td>6.0 (1.1–12.1)</td>
</tr>
<tr>
<td>3</td>
<td>13.3 (3.6–34.3)</td>
</tr>
<tr>
<td>4</td>
<td>19.5 (15.6–25.2)</td>
</tr>
</tbody>
</table>

⁶ Absolute cell number × 10⁶ was determined by multiplying the percentage of CD45R⁺ sIgM⁻ cells by total cell number for three independent cultures at each time point.

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**FIGURE 5.** TR expression during B cell development. The expression of TRα1, TRα2, TRβ1, and TRβ2 was determined on bone marrow B lineage cells sorted into Hardy fractions A, B + C, D, and E + F by RT-PCR and Southern blot analysis. Results are representative of one of three experiments.

**FIGURE 6.** Effect of T3 on the proliferation of hyt/hyt CD45R⁺ sIgM⁻ bone marrow cells. Proliferation was measured in medium containing FBS (■) or thyroid hormone-depleted FBS (□) supplemented with 10⁻⁸ M T3, IL-7, or T3 + IL-7. Data based on analysis of three wells per condition.
cells. In any case, this model provides a regenerative challenge to which B cell progenitors would not normally be subjected. As expected, the 5-FU treatment accentuated the proliferative deficiency of B lineage cells in hyt/hyt mice, because the average frequency of pro-B cells in the S-G2/M phases of the cell cycle was ~10\% lower than in their +/− littermates. To confirm that the lower frequency of cycling cells in the 5-FU-treated hyt/hyt mice was a result of thyroid hormone deficiency, additional groups of mice were treated with T4 after 5-FU administration. Consistent with its proposed role as a regulator of pro-B cell proliferation, the frequency of cycling CD45R^+CD43^+ pro-B cells in T4-treated hyt/hyt mice was on average 8\% above that in saline-treated hyt/hyt animals. Additionally, the absolute number of cycling pro-B cells in hyt/hyt mice treated with T4 was significantly higher than that in saline-treated mice. These results appear to explain why the frequency of B lineage cells was significantly increased in the T4-treated mice.

The increase in the frequency and absolute number of B lineage cells in the 5-FU/T4-treated hyt/hyt mice was accompanied by a decline in the frequency of CD45R^− cells. In one of these experiments, this decline in the frequency of CD45R^− cells was paralleled by a lower frequency of proliferating CD45R^− cells, which are presumably myeloid (data not shown). There is a well-established, reciprocal relationship between B lymphopoiesis and myelopoiesis (46), so these results are not surprising. Thus, as the number of B lineage cells increased as a result of T4 treatment, there was a proportionate decline in the number of myeloid cells. One possible explanation for this observation is that thyroid hormones might act, not by stimulating B lymphopoiesis, but by inhibiting the growth and/or differentiation of myeloid cells. This seems unlikely for two reasons. First, the addition of T3 to myeloid long term bone marrow cultures had no effect on cell production (data not shown). Additionally, exogenous thyroid hormone had no effect on the number of myeloid colonies generated in response to GM-CSF (data not shown). Taken together, these observations strongly suggest that the decline in the frequency and cycling status of CD45R^− cells in T4-treated hyt/hyt mice is not due to direct suppressive effects of thyroid hormones on non-B lineage cells.

To determine the potential for thyroid hormones to affect B lymphopoiesis through direct effects on the bone marrow, T3 was added to long term bone marrow cultures. In these experiments, the kinetics of the emergence of pre-B and B cells in the cultures was not affected. This result is consistent with the previous suggestion that thyroid hormones do not affect pro-B cell differentiation. However, the frequency and absolute number of B lineage cells were significantly higher in T3-supplemented cultures. Although these results do not exclude extramedullary actions of thyroid hormones, the data clearly demonstrated that at least some effects of thyroid hormones are directly mediated on the bone marrow. In addition, they support the observation that thyroid hormone stimulates proliferation of B lineage cells.

In view of these results, further studies were conducted to determine whether or not thyroid hormone affected B lineage cells directly. Initial experiments assessed whether or not the TR was expressed during B lymphopoiesis. TRs are members of a family of ligand-dependent, zinc-finger transcription factors (47, 48). They are encoded by two genes, α and β, and alternative splicing produces receptor isoforms. All TRs have DNA-binding domains, and except for TRα2, ligand-binding domains (49). Upon binding ligand, the receptor/ligand complex then binds DNA and activates gene expression. On the other hand, TRα2 cannot bind thyroid hormone, but it can still bind DNA and thus acts as a dominant-negative form of the receptor (50). Additionally, TRα2 has been shown to interfere with transcription activation of the active ligand-binding forms of the receptor (51, 52).

Published results have shown that TRα mRNA is expressed in chicken bone marrow cells (53) and murine B cell lines (54), but there have been no reports detailing TR expression during each stage of B lymphopoiesis. RT-PCR and Southern blot analysis showed that TRα1 and TRβ1 were constitutively expressed throughout B cell development, whereas TRβ2 was not detected. The absence of TRβ2 expression was not surprising because it has only been detected in the anterior pituitary and the brain (55, 56). The most striking result of this experiment was the differential regulation of TRα2 during B lymphopoiesis. The results show that TRα2 is expressed in fractions A–C, down-regulated at fraction D, and then up-regulated at fraction E + F. Interestingly, thyroid hormone-deficient mice have the greatest decrease in cell frequency and absolute number in fraction D (15). Whether the down-regulation of TRα2 during the fraction C to D transition is relevant to the dramatic cell loss at fraction D in thyroid hormone-deficient mice is currently being investigated.

The results of the long term culture experiments and the data showing that pro-B cells express TRs α and β provided a rationale for experiments to demonstrate a direct effect of T3 on B lineage cells. However, attempts to do so were unsuccessful. T3 did not stimulate the proliferation of sorted CD45R^+sgm− B lineage cells in vitro, nor did it act as an IL-7 proliferator cofactor. Taken together, the abilities to demonstrate direct growth-promoting effects or IL-7 cofactor activity suggest that thyroid hormones regulate the size of the pro-B cell compartment in an as yet uncharacterized manner.

The challenge will be to determine at the molecular level how thyroid hormones regulate pro-B cell growth. Thyroid hormone acts through its TRs to regulate gene expression (28). In the ligand-bound form, the TRs can activate transcription, whereas in the unbound form, they can repress it (57). Reduced levels of thyroid hormone could thus affect gene regulation by down-regulating expression of thyroid hormone-responsive genes. If these included genes that encoded cyclins and/or cyclin-dependent kinases in early B precursors, their proliferation could be compromised. Alternatively, the TR/thyroid hormone complex may control expression of regulatory proteins that in turn modulate the expression of molecules that inhibit the function of cell cycle proteins. For example, studies have shown that depletion of thyroid hormone inhibited proliferation of human glioblastoma cells and arrested them in G1 through up-regulation of p21, an inhibitor of cyclin-dependent kinases (58). Further studies will be needed to investigate whether a similar effect occurs in pro-B cells. Nevertheless, the data in this report provide an explanation for the decreased frequency of B lineage cells observed in thyroid hormone-deficient mice and further establish a role of the pituitary/thyroid axis in B cell development.

Acknowledgments

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References


