Abstract. We examined the effects of the long-term administration to mice of thyroid hormone or propylthiouracil (PTU) on lymphocyte subsets in spleens, and thymuses to clarify whether hyperthyroxinemia itself causes the changes in lymphocyte subsets, such as the marked increase in CD5+ B cells and decrease in natural killer (NK) cells, observed in hyperthyroid Graves' disease. Both the number and proportion of splenic NK (Thy-1+ asialo GM1+) cells were increased in hyperthyroxinemic mice treated with thyroxine (T4) for both short and long terms (8 and 32 weeks, respectively), those of splenic and thymic T (CD5+ sIgM-) cells were increased only in hyperthyroxinemic mice treated for 32 weeks, and those of splenic B (sIgM+) cells and CD5- B cells were increased only in hypothyroxinemic mice treated with PTU for 32 weeks, compared with those in euthyroid mice. These data indicate that 1) long-term hyperthyroxinemia increases splenic and thymic T cells and splenic NK cells, but not CD5+ B cells, in mice, 2) long-term hypothyroxinemia induced by PTU treatment increases splenic B cells and CD5- B cells, and 3) hyperthyroxinemia itself does not cause the changes in CD5+ B cells and NK cells, which are observed in hyperthyroid Graves' disease, in mice.

Key words: Thyroid hormone, Lymphocyte subsets, Spleen, Thymus, CD5+ B cells

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In this study, we examined the effects of the long-term administration to mice of thyroid hormone or propylthiouracil (PTU) on lymphocyte subsets including CD5+ B cells and NK cells to find whether hyper- or hypothyroxinemia itself causes such changes.

Materials and Methods

Mice and treatment

Female C57BL/6J mice (Thy-1.2), which do not develop spontaneous autoimmune disease, were obtained at 4 weeks of age from Clea Japan, Inc., Tokyo, Japan. After acclimatization for 3 weeks, the mice were given commercial chow pellets (CRF-1, Oriental Yeast Co., Tokyo, Japan) and 1 mg of thyroxine (T4, Sigma Chemical Co., St. Louis, MO) per liter of tap water containing 0.1% bovine serum albumin (BSA, Sigma) ad libitum for induction of hyperthyroxinemia, or else they received CRF-1 chow and 0.1% PTU (Nacalai Tesque, Inc., Kyoto, Japan) in tap water containing 0.1% BSA ad libitum for induction of hypothyroxinemia [1]. Control mice were provided with CRF-1 chow and tap water containing 0.1% BSA ad libitum. After 8 or 32 weeks of treatment, lymphocyte subsets were examined in each group of mice.

Preparation of splenic, thymic, and blood cells

Mice were killed by exsanguination or were anesthetized with ether. The spleen and thymus were removed and put into ice-cold phosphate-buffered saline (PBS, containing 0.1% BSA and 0.1% sodium azide). Single-cell suspensions of the spleen and thymus were prepared as follows. The organs were cut into pieces with scissors, crushed, passed through a 100-mesh stainless steel screen, and then passed through a 400-mesh stainless steel screen. After being washed with ice-cold PBS, the cells were suspended in ice-cold PBS at a cell concentration of 0.5 × 10⁷ to 1 × 10⁷/ml for peritoneal cells and 1 × 10⁷/ml for splenic and thymic cells. Blood was collected from some mice by cardiac puncture with a heparinized syringe before the injection of PBS under ether anesthesia. Blood cells were washed three times with ice-cold PBS.

Antibodies

Biotinated rat monoclonal antibodies to mouse CD5 (Ly-1; 53.7.3) and Thy-1.2 (30-H12) and streptavidin conjugated with phycoerythrin were obtained from Becton Dickinson & Co., Mountain View, CA; rat anti-mouse immunoglobulin M (IgM) monoclonal antibodies (μ-chain specific, LO-MM-9) conjugated with fluorescein isothiocyanate (FITC) were from Zymed Laboratories, San Francisco, CA; rabbit anti-asialo GM1 antibodies were from Wako Pure Chemical Industries, Osaka, Japan; and affinity-purified F(ab’)$_2$ fragments of goat anti-rabbit IgG antibodies (heavy- and light-chain-specific) conjugated with FITC were from Cappel Laboratories, West Chester, PA.

Analysis of lymphocyte subsets

For staining of CD5+ B cells, 100-µl samples of cell suspensions were incubated for 30 min at 4°C with biotinated anti-mouse CD5 antibodies and anti-mouse IgM monoclonal antibodies conjugated with FITC, and then washed twice with ice-cold PBS. The mixtures were next incubated for 30 min at 4°C with streptavidin conjugated with phycoerythrin and washed twice with ice-cold PBS. For staining of natural killer (NK) cells, 100-µl samples of cell suspensions were incubated for 30 min at 4°C with biotinated anti-mouse Thy-1.2 monoclonal antibodies and anti-asialo GM1 antibodies and washed twice with ice-cold PBS. The mixtures were next incubated for 30 min at 4°C with streptavidin conjugated with phycoerythrin and F(ab’)$_2$ fragments of anti-rabbit IgG antibodies conjugated with FITC, and then washed twice with ice-cold PBS. The stained cell samples were hemolyzed, fixed with lysing reagent (FACS lysing solution, Becton Dickinson), washed twice with ice-cold PBS, and suspended in 0.5 ml of PBS.

The samples were studied by two-color flow cytometry in an apparatus with an autocompensation system (FACScan; Becton Dickinson) to find the proportions of lymphocyte subsets as a percentage of total lymphocytes. The cells bearing CD5 but not surface IgM were regarded as T cells, the cells bearing surface IgM were regarded as B cells, and the cells bearing asialo GM1 and Thy-1 were regarded as NK cells [8, 21–23].
Measurement of serum levels of free T₄

Blood samples were collected from mice by cardiopuncture or puncture of the inferior vena cava under ether anesthesia, and sera were separated. Serum levels of free T₄ were measured with radioimmunoassay free T₄ kits for clinical use (Eiken Chemical Co., Tokyo, Japan; 24).

Statistical analysis

The statistical significance of differences was evaluated by Student’s t-test. When variances were unequal, the Mann-Whitney U-test was used.

Results

Serum levels of free T₄ were higher in mice treated with T₄ and lower in mice treated with PTU than in control mice treated with BSA only at both times assayed (Fig. 1).

The body weights in the hyper- and hypothyroxinemic mice treated for 8 and 32 weeks were not significantly different from those of the control mice (Fig. 1). However, the spleen weights increased in hyperthyroxinemic mice at both 8 and 32 weeks of treatment, and their thymus weights increased slightly, at 32 weeks of treatment only (Fig. 1). The weights of these organs in the hypothyroxinemic mice were not significantly different from those of the controls.

The proportions of lymphocyte subsets in splenic cells are shown in Table 1. The proportion of T cells had increased in hyperthyroxinemic mice and decreased in hypothyroxinemic mice at 32 weeks of treatment. The proportion of B cells and CD5⁻ B cells had decreased in hyperthyroxinemic mice at 32 weeks of treatment, and had increased in hypothyroxinemic mice treated for 32 weeks. The proportion of splenic CD5⁺ B cells had not changed.

![Fig. 1.](image-url) Serum levels of free T₄ and weights of body, spleen, and thymus in control (■), hyperthyroxinemic (●), and hypothyroxinemic (▲) mice treated with BSA, T₄, or PTU, respectively. Results are presented as the mean ± SD (bars) for 7 mice. *P<0.05, **P<0.01, and ***P<0.001 vs. control mice.)
in hyper- and hypothyroxinemic mice treated for 8 and 32 weeks. The proportion of NK cells increased in hyperthyroxinemic mice treated for 8 and 32 weeks, and decreased in hypothyroxinemic mice treated for 32 weeks. The proportion of NK cells in the blood was examined only in mice treated for 32 weeks. It had increased in hyperthyroxinemic mice (n=7, 6.5 ± 0.5%, P<0.001) and decreased in hypothyroxinemic mice (n=7, 2.5 ± 0.5%, P<0.05) compared with control mice (n=7, 3.1 ± 0.3%).

The proportions of lymphocyte subsets in thymic cells are shown in Table 2. The proportions of T, B, CD5+ B, CD5− B, and NK cells had not changed in either hyper- or hypothyroxinemic mice at either 8 or 32 weeks of treatment, except for a slight increase in the proportion of thymic T cells in hyperthyroxinemic mice at 32 weeks of treatment. In hyperthyroxinemic mice treated for 32 weeks, the absolute numbers of splenic T cells and NK cells were greater than those in control mice (Fig. 2), but the numbers of splenic B cells, CD5+ B cells, and CD5− B cells were not significantly different. In hypothyroxinemic mice treated for 32 weeks, the absolute numbers of B cells and CD5− B cells had increased (Fig. 2), but the numbers of the other cells assayed were not significantly different. No significant changes were observed in the numbers of these lymphocyte subsets in thymic cells from hyper- and hypothyroxinemic mice at 32 weeks of treatment, except for an increase in the number of thymic T cells in hypothyroxinemic mice (Table 3). Furthermore, no significant changes were observed in the number of these lymphocyte subsets in splenic or thymic cells from hyper- and hypothyroxinemic mice at 8 weeks of treatment, except for an increase in the number of splenic NK cells [n=7, (3.9 ± 0.7) x 10⁶/spleen, P<0.01] in hyperthyroxinemic mice, as compared with that in control mice [n=7, (2.3 ± 0.4) x 10⁶/spleen].

**Discussion**

One group has reported that the proportions of splenic and blood T cells do not change in rats treated with triiodothyronine or PTU for 17 days [14], but another group reports that the proportion of blood T cells decreases in hyperthyroxinemic rats treated with T₄ for 5 weeks [18]. Our results show that hyper- and hypothyroxinemia for 8
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weeks did not change the proportions and numbers of T and B cells in the spleen or thymus, and that only hyperthyroxinemia for 8 weeks increased the proportion and number of NK cells in the spleen. Most of the changes in splenic lymphocyte subsets occurred when hyper- and hypothyroxinemia were long-term. Hyperthyroxinemia that lasted 32 weeks (which is considerably longer than 6 months) increased the proportions and numbers of T cells and NK cells and decreased the proportions of B cells and CD5- B cells in the spleens of mice. Long-term hypothyroxinemia increased the numbers and proportions of B cells and CD5- B cells and decreased the proportions of T cells and NK cells in the spleens of mice. Long-term hyper- and hypothyroxinemia did not affect these lymphocyte subsets in the thymus, except for a slightly increased number and proportion of thymic T cells in hyperthyroxinemic mice. These findings indicate that long-term hyper- and hypothyroxinemia are required to change the numbers of T, B, and CD5- B cells in hyperthyroxinemic mice. This increase in T cells may be caused by thymulin, the production of which is increased by thyroid hormones [17], and may be related to an increase in the blastogenic response of thymic and peripheral lymphocytes to T cell mitogens in hyperthyroxinemic rats [15]. The increase in NK cells in hyperthyroxinemia may cause the decrease in antibody production found in hyperthyroxinemic rats [12, 16], because NK cells reduce antibody production by killing activated B cells [25]. As for NK activity, the effect of hyperthyroxinemia in mice is controversial [10, 11], and also no consistent result was obtained in hyperthyroid patients with Graves’ disease [26, 27].

Long-term hyperthyroxinemia did not cause the changes in lymphocyte subsets observed in hyperthyroid Graves’ disease, that is, an increase in CD5+ B cells and a decrease in NK cells [6]. Furthermore, we also examined the changes in lymphocyte subsets in the peritoneal cavity where CD5+ B cells

Table 2. Changes in percentages of thymic T, B, and NK cells in control, hyperthyroxinemic and hypothyroxinemic mice treated with BSA, T4 or PTU, respectively

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hyperthyroxinemia</th>
<th>Hypothyroxinemia</th>
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<tbody>
<tr>
<td></td>
<td>T cells</td>
<td>B cells</td>
<td>CD5- B</td>
</tr>
<tr>
<td></td>
<td>97.2 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>97.3 ± 0.5</td>
<td>1.1 ± 0.6</td>
<td>0.1 ± 0.0</td>
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<tr>
<td></td>
<td>94.5 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>0.2 ± 0.1</td>
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<tr>
<td></td>
<td>97.6 ± 0.7</td>
<td>1.1 ± 0.8</td>
<td>0.1 ± 0.1</td>
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<tr>
<td></td>
<td>95.7 ± 0.8**</td>
<td>1.2 ± 0.5</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>97.5 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>94.9 ± 0.4</td>
<td>1.0 ± 0.4</td>
<td>0.2 ± 0.1</td>
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</tbody>
</table>

Results are the mean ± SD for 7 mice. BSA, bovine serum albumin; T4, thyroxine; PTU, propylthiouracil; NK, natural killer. **P<0.01 vs. control mice.
may be produced and are abundant [9], but we did not find significant changes (data not shown). Thus, such changes in lymphocyte subsets in hyperthyroid Graves' disease may not be caused by hyperthyroxinemia itself. This discrepancy may be explained by another possibility: that stimulation of thyroid epithelial cells with TSH-receptor antibodies is related to these changes in Graves' disease. Thyroid epithelial cells produce cytokines such as interleukins-1 and -6 [28, 29] as well as thyroid hormones. Some growth factor for CD5+ B cells, such as interleukin-5 [30], may therefore be produced by thyroid epithelial cells also, and its production may be increased in stimulated thyroid cells. However, thyroid epithelial cells are resting (not stimulated) in hyperthyroxinemic mice treated with T4.

In this study, we induced hypothyroxinemia in mice by treatment with PTU as in a previous study [14], although it has been reported that PTU suppresses the immune system [3]. Long-term hypothyroxinemia induced by PTU treatment increased total B cells and CD5- B cells, but not T cells and NK cells, in the spleens of mice. However, short-term hypothyroxinemia did not change these lymphocyte subsets, as reported previously [14]. It is possible that TSH, which is increased in hypothyroxinemia, increases B cells, since TSH stimulates B cells and increases antibody production [31], but changes in B cells and CD5- B cells are not significant in hypothyroid patients with Hashimoto's disease [5, 7]. One explanation of these differences is that the severity or duration of hypothyroidism in the mice examined in this study

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**Table 3. Absolute numbers of thymic lymphocyte subsets in control, hyperthyroxinemic and hypothyroxinemic mice treated with BSA, T4 or PTU, respectively, for 32 weeks**

<table>
<thead>
<tr>
<th>Thymic cells</th>
<th>after 32 w</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(× 10⁶/thymus)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>46.7 ± 6.3</td>
</tr>
<tr>
<td>B cells</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>CD5- B</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>CD5+ B</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Hyperthyroxinemia</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>60.7 ± 4.9**</td>
</tr>
<tr>
<td>B cells</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>CD5- B</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>CD5+ B</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Hypothyroxinemia</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>53.1 ± 7.7</td>
</tr>
<tr>
<td>B cells</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>CD5- B</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>CD5+ B</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.7 ± 0.2</td>
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</tbody>
</table>

Results are the mean ± SD for 7 mice. BSA, bovine serum albumin; T4, thyroxine; PTU, propylthiouracil; NK, natural killer. ** P<0.01 vs. control mice.
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may be greater or longer than that in the patients with Hashimoto's disease.

The weights of the spleen and thymus increased in hyperthyroxinemic mice treated with T4 in this study, as found by other investigations [11, 14, 18], although the increase in thymic weight was slight and took longer. An excess of thyroid hormone itself is therefore one factor that causes the enlargement of these organs, especially the spleen, often observed in hyperthyroid patients with Graves' disease [32].

In conclusion, hyperthyroxinemia itself does not cause the changes in CD5+ B cells and NK cells, which are observed in hyperthyroid Graves' disease, in mice.

Acknowledgments

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References


