Immune Development in Young-Adult C.RF-*hyt* Mice Is Affected by Congenital and Maternal Hypothyroidism (43632)

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Abstract. **C.RF-*hyt* mice carry a mutation (*hyt*) that results in the phenotypic expression of congenital hypothyroidism in *hyt/hyt* mice due to a nonresponsiveness of the thyroid gland to thyroid-stimulating hormone. Heterozygotes of this strain are euthyroid. To further define thyroid-immune interactions, the effect of congenital hypothyroidism and maternal hypothyroidism on immune development were examined in 3- to 4-month-old *hyt/+* and *hyt/hyt* progeny from *hyt/+* and *hyt/hyt* dams.**

The state of immune development in these mice was compared on the basis of immune organ weights and the proliferation response of splenocytes stimulated with the T cell mitogens concanavalin A (Con A) and phytohemagglutinin and the B cell mitogen lipopolysaccharide. In addition, analysis of T cell subpopulations in thymus and spleen was conducted using direct and indirect immunofluorescence and flow cytometry.

Data analyses for the main effects of congenital hypothyroidism on immune development revealed a significantly lower absolute thymus weight ($P < 0.001$), a lower ($P = 0.022$) percentage of thymocytes expressing CD8 ($CD8^+$), a higher ($P = 0.010$) ratio between $CD4^+$ and $CD8^+$ thymocytes, a lower ($P < 0.001$) absolute and adjusted spleen weight, a lower ($P = 0.001$) Con A to phytohemagglutinin response ratio, a higher ($P = 0.003$) percentage of $CD4^+$ splenocytes, and a marginally significant ($P = 0.055$) increase in the ratio between $CD4^+$ and $CD8^+$ splenocytes in hypothyroid compared with euthyroid mice.

Data analyses for the main effects of maternal hypothyroidism revealed a significantly higher absolute ($P = 0.025$) and adjusted ($P = 0.001$) thymus weight, a higher ($P = 0.006$) ratio between $CD4^+$ and $CD8^+$ thymocytes, a lower Con A ($P = 0.018$) and lipopolysaccharides ($P < 0.001$) response, a marginally ($P = 0.069$) lower Con A to phytohemagglutinin response ratio, a lower ($P = 0.001$) percentage of $CD4^+$ splenocytes, and a lower ($P = 0.003$) ratio between $CD4^+$ and $CD8^+$ splenocytes in progeny of hypothyroid compared with progeny of euthyroid mothers.

These data provide further evidence for the importance of normal thyroid function in the development, maintenance, and function of the immune system. It was concluded that not only congenital hypothyroidism results in altered immune development in young-adult mice, but also long-term effects on immune development occur in progeny of hypothyroid mothers. [P.S.E.B.M. 1993, Vol 204]

The importance of normal thyroid function and circulating levels of iodothyronines for the development and function of the immune system has been demonstrated in mammalian (1–3) and avian species (4–6). Nevertheless, there is still relatively little known about the extent and mechanism of this interrelationship. Effects of maternal hypothyroidism on immune development in her offspring have, to our knowledge, not been examined.

There are a variety of animal models available to study the effects of low iodothyronine state on immune development. These include animals rendered hypothyroid by thyroidectomy (7) or by administration of propylthiouracil (2) and animal models with congenital hypothyroidism, such as the sex-linked dwarf chicken (4, 5, 8), the hypopituitary *dw/dw* Snell-Bagg dwarf mouse (9, 10), and the C.RF-*hyt* mouse, which has

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never been used to study thyroid-immune interactions. Mice from the C.RF-*hyt* strain that are homozygous recessive for the *hyt* gene are born hypothyroid, with serum thyroxine levels below the limit of detection (11), and are dwarfs, whereas heterozygotes (*hyt*/+) and wild-type (+/+) mice are euthyroid and have normal body size. The hypothyroidism is already present at Day 18 of ontogenic development and is due to the unresponsiveness of the thyroid gland to thyroid-stimulating hormone (11, 12). Recent studies by Stein *et al.* (13) have shown that the unresponsiveness of the thyroid gland to thyroid-stimulating hormone is apparently due to a defect in the adenylyl cyclase system in the *hyt/hyt* thyroid gland. This defect at the level of the thyroid gland makes the *hyt/hyt* model different from the hypothyroidism observed in the hypopituitary dwarf mouse, which has an insufficiency at the level of the anterior pituitary gland (14), and from the sex-linked dwarf chicken, which has low triiodothyronine levels in the blood due to a defect in the peripheral conversion of thyroxine to triiodothyronine (15). Like other animal models for congenital hypothyroidism, the *hyt/hyt* mouse has the advantage that no invasive manipulations, such as thyroidectomy or treatment with propylthiouracil, are required to induce hypothyroidism. The C.RF-*hyt* mouse strain has the additional advantage that it is inbred and shares its background genes, including the major histocompatibility complex ($H\text{-}2^d$), with the immunologically well-studied BALB/cByJ mouse. Lastly, we found *hyt/hyt* females to be able to have a limited number of litters without thyroxine supplementation and thus provide an excellent opportunity to examine the effects of maternal hypothyroidism on immune development and function.

The purpose of this experiment was to gain insight into the state of immune development and function in young-adult euthyroid (*hyt*/+) and hypothyroid (*hyt/hyt*) C.RF-*hyt* progeny from euthyroid (*hyt*/+) and hypothyroid (*hyt/hyt*) dams. Immune aspects examined included determination of immune organ weights, the proliferative response of splenocytes to stimulation with the T cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) and the B cell mitogen lipopolysaccharide (LPS), and analysis of T cell subpopulations in the thymus and spleen. It was determined that both congenital and maternal hypothyroidism affect various aspects of immune development.

Materials and Methods

Experimental Animals. Euthyroid (*hyt*/+) and hypothyroid (*hyt/hyt*) C.RF-*hyt* mice (Jackson Laboratories, Bar Harbor, ME) were bred and maintained in the animal facilities at Smith College, Northampton, MA. Breeder mice were fed mouse breeder chow (Old Guilford Mouse Diet No. 911 R; Emory Morse Co., Guilford, CT). Experimental animals were maintained on mouse chow (Autoclavable Wayne Rodent Blox, Teklad Premier; Laboratory Diets, Madison, WI). All mice received food and water *ad libitum*. Mice were housed in plastic shoe box cages (18.5 in L × 10 in W × 8 in H) at 23°C and a 12:12-hr light:dark cycle. To obtain experimental animals, two types of matings were set up. In Group 1, euthyroid (*hyt*/+) female C.RF-*hyt* mice were mated with hypothyroid (*hyt/hyt*) males that had been fed breeder chow meal supplemented with 0.025% desiccated thyroid powder before they were used for mating. In Group 2, hypothyroid (*hyt/hyt*) female C.RF-*hyt* mice were mated with euthyroid (*hyt*/+) males. At 4 weeks of age, the offspring were separated from their mothers and their sex and thyroid state were determined. Euthyroid (*hyt*/+) and hypothyroid (*hyt/hyt*) offspring were distinguished on the basis of the much smaller body size and skimpy fur of the *hyt/hyt* mouse, which is clearly noticeable at this age and at the time of the experiment (see Results). In concurrent studies (unpublished), we have confirmed that this method of selecting *hyt/hyt* mice is extremely reliable, and every mouse identified as hypothyroid by this method was confirmed to be hypothyroid by radioimmunoassay (clinical assays, total T_4 radioimmunoassay; Incstar Corp., Stillwater, MN). Male and female offspring were separated and assigned to separate plastic cages two to three mice/cage.

Experiment I. Three- to 4-month-old *hyt*/+ (20 mice) and *hyt/hyt* (20 mice) offspring from Group 1 matings and *hyt*/+ (20 mice) and *hyt/hyt* (19 mice) offspring from Group 2 matings were euthanized with CO_2-gas. Each treatment group contained equal or near equal (10 vs 9) numbers of males and females. The animals were weighed and their spleen and thymus were removed under sterile conditions. Mediastinal lymph nodes and any adhering fat were teased away from the thymus and fresh weights of thymus and spleen were determined for each mouse (absolute thymus and spleen weight). In addition to absolute immune organ weights, thymus and spleen weights were also adjusted for body weight differences and expressed as mg/100 g body wt when comparisons were made between treatment groups (adjusted thymus and spleen weight).

Splenocyte Proliferation Assays. To examine the proliferative response of splenocytes to stimulation with T cell and B cell mitogens, splenocyte suspensions were prepared by gently meshing the spleen through a 60-μm nylon screen with a sterile syringe plunger into a sterile beaker with frequent addition of cold Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO). The cell suspensions were washed at 4°C, 180*g*, for 10 min and erythrocytes were then lysed with Tris-buffered ammonium chloride (16). After erythrocyte lysis splenocytes were washed twice more as before and cultured in complete medium (CM) consisting of 100

ml of RPMI 1640 medium supplemented with 2 ml of heat-inactivated fetal bovine serum, 1 ml of glutamine (200 m*M*), 1 ml of 5×10^{-3} 2-mercaptoethanol, and 1 ml of penicillin-streptomycin (10,000 units penicillin/ml and 10 mg streptomycin/ml). For each mouse, 2×10^5 cells/well in 0.1 ml of CM were cultured with various concentrations of Con A (0.3, 0.2, 0.1, and 0 μg/well in 0.1 ml of CM), PHA-P (4, 2, 1, and 0 μg/well in 0.1 ml of CM), and LPS (4, 2, 1, and 0 μg/well in 0.1 ml of CM). The duration of culture and the range of mitogen concentrations were predetermined by exposing splenocytes from each treatment group to a wide range of mitogen concentrations and incubation times. A 72 hr, incubation time and 0.2 μg Con A/well, 2.0 μg PHA-P/well, and 2.0 μg LPS/well were found to yield an optimal mitogen response independent of genotype and gender of the offspring and genotype of the mother. All mitogens and culture materials were purchased from Sigma. Three wells of a 96-well round-bottom culture plate were set up for each mitogen concentration. The cultures were incubated in a CO_2-controlled, humidified incubator at 37°C for 72 hr. One μCi [^{3}H]thymidine/well (sp act 2 Ci/mmol; ICN Radiochemicals, Irvine, CA) in 25 μl of CM was added to the cultures during the last 24 hr of incubation. The cells were harvested onto glass-fiber filters and the uptake of [^{3}H]thymidine was determined in a scintillation counter (Beckman LS 7500; Beckman Instrument Inc., Irvine, CA). The data were expressed as cpm in mitogen-stimulated cultures minus cpm in unstimulated cultures (0 μg of mitogen). The ratio between the proliferation of splenocytes in response to stimulation with Con A versus that with PHA (Con A to PHA ratio) was calculated by dividing cpm of the Con A response by cpm of the PHA response for each individual.

Experiment II. Thymuses and spleens from 3- to 4-month-old *hyt/+* (13 mice) and *hyt/hyt* (11 mice) offspring from Group 1 matings and *hyt/+* (six mice) and *hyt/hyt* (12 mice) offspring from Group 2 matings were obtained as described in Experiment I. To prepare single-cell suspensions, the thymuses were gently meshed through a 60-μm nylon mesh and washed three times in Hanks' balanced salt solution as before. Single-cell suspensions of spleens were prepared as described in Experiment I. After the final wash, pellets of thymocytes and splenocytes were resuspended in 0.25 ml of staining medium (SM; Hanks' balanced salt solution without a pH indicator, containing 0.1% sodium azide and 2% goat serum; Sigma). For direct and indirect immunofluorescent labeling, 2×10^6 cells in 20 μl of SM were added to 12 × 75 Falcon tubes. For direct, double-fluorescent staining, cells were incubated for 30 min at 4°C with 3 μl of undiluted fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8 monoclonal antibody and 3 μl of undiluted phycoerythrin (PE)-conjugated rat anti-mouse CD4 monoclonal antibody (Becton-Dickinson Immunocytochemistry Systems, Mountain View, CA; anti-lyt-2-FITC and anti-L3T4-PE, respectively), washed twice with cold SM, and resuspended in 1 ml of cold SM. For indirect, single-fluorescent staining of thymocytes, cell suspensions were incubated for 30 min at 4°C with 20 μl of a 1/50 dilution of mouse anti-mouse Thy1.2 monoclonal antibody (Sigma), washed in cold SM, and incubated with 20 μl of a 1/10 dilution of FITC-conjugated goat anti-mouse IgM antibody for 30 min at 4°C. Thymocytes were then washed twice with cold SM and resuspended in 1 ml of SM. All antibodies were deaggregated by centrifugation in a Savant high speed microcentrifuge (Savant Instruments Inc., Hicksville, NY) for 10 min at 9,000*g* before use and cell suspensions were kept on ice until flow cytometric analysis. Controls included thymocytes and splenocytes incubated with SM only (negative control), with FITC-conjugated rat IgG of irrelevant specificity (control for nonspecific binding, direct-staining method), with either FITC-conjugated rat anti-mouse CD8 monoclonal antibody or PE-conjugated rat anti-mouse CD4 monoclonal antibody (to adjust compensation for flow cytometric analysis), and with SM followed by FITC-conjugated goat anti-mouse IgM (nonspecific binding of conjugated antibody, indirect-staining method). The latter control also was used to ascertain that mediastinal lymph nodes present on the thymus gland were removed completely. Incubation procedures for controls were exactly as outlined above for direct and indirect immunofluorescent staining. Cell population analysis was then carried out by flow cytometry using a FACScan with Consort 30 software package (Becton Dickinson Immunocytochemistry Systems). The parameter gains were adjusted to place single viable cells in the first quarter of the scale using the negative control samples. Two-color compensation was set using single-label controls. Debris and dead cells were excluded from the analysis on the basis of low-angle light scatter and/or propidium iodide uptake. Data were acquired in list mode and 10,000 viable cells were examined for each sample. The dot cloud obtained when examining nonspecific binding control samples was used as a guide to distinguish fluorescence-negative, PE^+, $FITC^+PE^+$, and $FITC^+$ cells for each mouse and tissue. Data were expressed as percent Thy1.2$^+$, percent $CD4^+CD8^-$ ($CD4^+$), percent $CD4^+CD8^+$, or percent $CD4^-CD8^+$ ($CD8^+$) thymocytes and percent $CD4^+$ or percent $CD8^+$ splenocytes. The CD4 to CD8 ratio was calculated by dividing the percentage of $CD4^+$ cells by the percentage of $CD8^+$ cells for each cell type and each individual.

Statistical Analysis. All statistical analyses were carried out using the SYSTAT statistical analysis computer package (Systat, Inc., Evanston, IL). For splenocyte proliferation assays, analysis of variance was used

to test for the effects of mitogen concentration, congenital hypothyroidism (CH), maternal hypothyroidism (MH), gender, and the interaction among these variables on the proliferative response of splenocytes to Con A, PHA, and LPS. Separate analyses of variance were conducted to test for the effects of CH, MH, gender, and their interactions on each of the other response variables: body weight, absolute and adjusted thymus and spleen weights, Con A to PHA ratio, percent Thy1.2$^+$, percent CD4$^+$, percent CD8$^+$CD4$^+$ and percent CD8$^+$ thymocytes, percent CD4$^+$ and percent CD8$^+$ splenocytes, and the CD4 to CD8 ratio for thymocytes and splenocytes. All analyses were computed using Systat's Multivariate General Linear Hypothesis procedure, which computes results for unbalanced study designs. Partial F tests were used to determine the significance of main effects and of their interactions. Test results were considered significant if $P < 0.05$.

Results

For the three mitogens examined, mitogen concentration had no significant effect on the proliferative response of splenocytes and there were no significant interactions between mitogen concentration and any other variables. Therefore, for each mitogen, the data for the three concentrations were averaged together. Partial F tests revealed that for most aspects of immune development examined, with the exception of body weights, there were no significant interactions between variables in both experiments. In the absence of interactions between gender and any of the other variables, data for males and females were pooled to increase the sample size when main effects of congenital hypothyroidism and maternal hypothyroidism were calculated. Hence, for Experiment I, the model that best described the main effect of CH (euthyroid versus hypothyroid) and MH (euthyroid versus hypothyroid dam) was CH with 1 degree of freedom (df), MH with 1 df, and error with 76 df for all immune aspects examined. Because of a gender × CH interaction for body weight, the main effect of CH and MH on body weight was examined separately for males and females. For Experiment II, the model that best described the main effects of CH and MH was CH with 1 df, MH with 1 df, and error with 39 df.

Main Effects of Congenital Hypothyroidism on Immune Development. Hypothyroid (*hyt/hyt*) females (17.89 ± 0.49 g) and hypothyroid males (19.18 ± 0.52 g) had a significantly ($P < 0.001$ and $P < 0.001$, respectively) lower mean body weight ± SE than euthyroid (*hyt/+*) females (23.16 ± 0.47 g) and males (27.56 ± 0.58 g). Absolute thymus weight was significantly lower in hypothyroid than in euthyroid mice. There was no significant difference between adjusted thymus weight in hypothyroid mice compared with that in euthyroid mice. Absolute and adjusted spleen weight was significantly lower in hypothyroid than in euthyroid mice (Table I).

The Con A, PHA, and LPS responses were not significantly different in hypothyroid compared with euthyroid mice. However, hypothyroid mice had a significantly lower Con A to PHA ratio than euthyroid mice (Table I).

No difference in percent Thy1.2$^+$, percent CD4$^+$, and percent CD4$^+$CD8$^+$ thymocytes were observed between hypothyroid and euthyroid mice. The percentage of CD8$^+$ thymocytes was significantly lower in thymuses from hypothyroid compared with euthyroid mice. The thymocyte CD4 to CD8 ratio was significantly higher in hypothyroid compared with euthyroid mice. In the spleen of hypothyroid mice, the percentage of CD4$^+$ splenocytes was significantly higher and the CD4 to CD8 ratio was marginally ($P = 0.055$) higher than in euthyroid mice. Differences in percent CD8$^+$ splenocytes were not significant when hypothyroid and euthyroid mice were compared (Table I).

Main Effect of Thyroid State of the Dam on Immune Aspects of Her Progeny. Body weight ± SE of female progeny of hypothyroid dams (20.12 ± 0.84 g) was not significantly ($P = 0.927$) different from that of female progeny of euthyroid mothers (20.94 ± 0.69 g). Body weight ± SE of male offspring from hypothyroid dams (22.28 ± 1.01 g) also was not significantly ($P = 0.137$) different from that of males with euthyroid mothers (24.75 ± 1.24 g). However, absolute and adjusted thymus weight were significantly higher in offspring of hypothyroid dams than in those of euthyroid mothers. No significant differences in absolute and adjusted spleen weight in progeny of hypothyroid and euthyroid dams were noted (Table II).

The Con A and LPS response of splenocytes were significantly lower in progeny of hypothyroid dams compared with progeny of euthyroid dams. There was no significant difference in the PHA response of splenocytes and only a marginally lower Con A to PHA ratio in progeny of hypothyroid dams compared with that of euthyroid dams (Table II).

The percentages of Thy1.2$^+$, CD4$^+$, CD4$^+$CD8$^+$, and CD8$^+$ thymocytes in offspring with hypothyroid mothers were not significantly different from those in offspring with euthyroid mothers. However, the thymocyte CD4 to CD8 ratio was significantly higher in progeny of hypothyroid dams than in progeny of euthyroid dams. The percentage of CD4$^+$ splenocytes and the splenocyte CD4 to CD8 ratio were significantly lower in offspring with hypothyroid mothers than in offspring with euthyroid mothers. No significant difference in percent CD8$^+$ splenocytes was observed when progeny of hypothyroid mothers and euthyroid mothers were compared (Table II).

Table I. Main Effects (Mean ± SE) of Congenital Hypothyroidism on Various Immune Aspects in 3- to 4-Month-Old Euthyroid (*hyt/+*) and Hypothyroid (*hyt/hyt*) Mice

Immune aspect	Mean response ± SE		*P*
	Euthyroid mice ($n = 40$)[a]	Hypothyroid mice ($n = 39$)	
Thymus wt (g)	0.028 ± 0.001	0.019 ± 0.001	0.000*
Thymus wt (mg/100 g body wt)	113 ± 5	105 ± 6	0.373
Spleen wt (g)	0.087 ± 0.003	0.046 ± 0.002	0.000*
Spleen wt (mg/100 g body wt)	343 ± 12	247 ± 10	0.000*
Con A response (cpm × 10^{-3})	75.6 ± 5.9	64.6 ± 6.1	0.178
PHA response (cpm × 10^{-3})	22.3 ± 2.0	23.8 ± 2.0	0.578
LPS response (cpm × 10^{-3})	23.7 ± 2.5	21.2 ± 2.1	0.410
Con A to PHA ratio[d]	3.78 ± 0.30	2.74 ± 0.16	0.001*
Thymocytes[e]			
Thy1.2$^+$ (%)	95.0 ± 0.5	93.9 ± 0.7	0.248
CD4$^+$ (%)	14.4 ± 1.0	16.9 ± 0.7	0.682
CD4$^+$CD8$^+$ (%)	73.7 ± 1.7	73.9 ± 1.1	0.944
CD8$^+$ (%)	5.8 ± 0.5	4.3 ± 0.3	0.022*
CD4 to CD8 ratio[d]	3.2 ± 0.2	4.6 ± 0.4	0.010*
Splenocytes[e]			
CD4$^+$ (%)	30.0 ± 1.4	36.5 ± 1.6	0.003*
CD8$^+$ (%)	17.8 ± 1.1	18.3 ± 1.0	0.756
CD4 to CD8 ratio[d]	1.8 ± 0.1	2.0 ± 0.1	0.055

[a] n = number of mice.
[b] $P < 0.05$ indicates a significant difference as shown by the asterisk.
[d] Average of individual ratios.
[e] Euthyroid ($n = 19$) and hypothyroid ($n = 23$).

Table II. Main Effects (Mean ± SE) of Maternal Hypothyroidism on Various Immune Aspects in 3- to 4-Month-Old Progeny of Euthyroid (*hyt/+*) and Hypothyroid (*hyt/hyt*) Dams

Immune aspect	Mean response ± SE		P^b
	Euthyroid dam ($n = 40$)[a]	Hypothyroid dam ($n = 39$)	
Thymus wt (g)	0.022 ± 0.001	0.026 ± 0.001	0.025*
Thymus wt[c]	94 ± 6	124 ± 6	0.001*
Spleen wt (g)	0.068 ± 0.004	0.065 ± 0.004	0.389
Spleen wt[c]	292 ± 14	298 ± 13	0.597
Con A response[d]	82.0 ± 6.8	58.3 ± 4.7	0.018*
PHA response[d]	25.3 ± 2.3	20.9 ± 1.5	0.105
LPS response[d]	27.4 ± 2.6	17.6 ± 1.7	0.000*
Con A to PHA ratio[e]	3.54 ± 0.31	2.98 ± 0.18	0.069
Thymocytes[f]			
Thy1.2$^+$ (%)	94.4 ± 0.4	94.6 ± 0.8	0.842
CD4$^+$ (%)	16.2 ± 0.8	18.0 ± 0.9	0.682
CD4$^+$CD8$^+$ (%)	74.1 ± 1.3	73.6 ± 1.4	0.800
CD8$^+$ (%)	5.5 ± 0.4	4.6 ± 0.5	0.146
CD4 to CD8 ratio[e]	3.1 ± 0.2	4.6 ± 0.5	0.006*
Splenocytes[f]			
CD4$^+$ (%)	37.0 ± 1.6	29.6 ± 1.1	0.001*
CD8$^+$ (%)	17.9 ± 1.0	18.3 ± 1.2	0.812
CD4 to CD8 ratio[e]	2.1 ± 0.1	1.7 ± 0.1	0.003*

[a] n = Number of mice.
[b] $P < 0.05$ indicates a significant difference as shown by the asterisk.
[c] mg/100 g of body wt.
[d] cpm × 10^{-3}.
[e] Average of individual ratios.
[f] Euthyroid ($n = 24$) and hypothyroid ($n = 18$).

Discussion

The data obtained in these experiments demonstrate that both congenital hypothyroidism and maternal hypothyroidism can influence various aspects of immune development in young-adult mice.

Effects of Congenital Hypothyroidism. A reduction in adjusted thymus weight after thyroidectomy has consistently been reported in the literature (6, 7). No differences in adjusted thymus weight between congenitally hypothyroid *hyt/hyt* and euthyroid *hyt/+* mice (Table I) were, however, observed here. Congenital hypothyroidism, unlike thyroidectomy, is not associated with an abrupt and dramatic drop in thyroid hormone concentration. As a possible consequence, mechanisms might develop to compensate for low thyroid function during development, which could explain the lack of differences in adjusted thymus weight between *hyt/hyt* and *hyt/+* mice. Alternatively, Fabris *et al.* (10) reported that the reduced adjusted thymus weight associated with low iodothyronine state was age dependent and was no longer observed in *dw/dw* Snell-Bagg mice compared with their normal siblings at 90 days of age. Similarly, adjusted thymus weight of male peripherally hypothyroid, sex-linked dwarf strain chickens compared with that of control K strain chickens was lower in 5-week-old chicks (17) but not in 12-week-old chicks (18). Thus, in young-adult euthyroid (*hyt/+*) mice, the thymus, which normally starts to atrophy after puberty (approximately 2 months of age), might have regressed to the thymic size of hypothyroid (*hyt/hyt*) mice, closing the gap in size between an already smaller, perhaps more slowly regressing, thymus and a larger thymus that had been regressing at a normal rate. Differences in the cellular composition of thymuses from *hyt/hyt* and *hyt/+* mice were, however, observed. Examination of the percentages of thymocytes expressing either Thy1.2 (T cells), both CD4 and CD8 (dual-positive, immature thymocytes), only CD4 (T helper cells), or only CD8 (T cytotoxic/suppressor cells) revealed approximately 25% fewer thymocytes expressing CD8 in hypothyroid compared with euthyroid mice. The reduction in the percentage of cells expressing CD8 was reflected in a 40% greater ratio of cells expressing CD4 versus those expressing CD8 (CD4 to CD8 ratio) in hypothyroid compared with euthyroid mice. Altered proportions of thymocyte populations were also observed by Johnson *et al.* (6) in 5-week-old, neonatally thyroidectomized chicks that had a relative increase in thymocytes expressing CD4. They suggested that the greater sensitivity of CD4 to thyroidectomy could be due to differences in the temporal expression of CD4 and CD8 by differentiating thymocytes, which also could explain the lower proportion of $CD8^+$ thymocytes in *hyt/hyt* mice observed here. Despite their differences, the data reported here and by Johnson *et al.* (6) strongly support the immunomodulatory effects of iodothyronines on T cell subsets in the thymus. Alterations in the normal maturation and differentiation of thymocytes, and hence in the balance among functionally distinct T cell subsets in the thymus, could have a considerable impact on the function of the immune system once mature T cells enter into the periphery.

In the spleen, a peripheral lymphoid organ, a marginal increase in the CD4 to CD8 ratio ($P = 0.055$), and a significant increase in percent $CD4^+$ splenocytes was observed in hypothyroid compared with euthyroid mice (Table I). Similar observations, an increase in percent $CD4^+$ splenocytes and in the splenocyte CD4 to CD8 ratio, were made by Pacini *et al.* (2) in spleens from thyroidectomized rats. However, in the blood of these thyroidectomized rats, they observed an increase in $CD8^+$ T cells and a decrease in the CD4 to CD8 ratio (2). The complementary changes in the proportions of T cell subpopulations in spleen and blood observed by Pacini *et al.* (2) suggest alterations in the circulation pattern of lymphocytes in addition to the dependency of T cell development on normal thyroid function.

The polyclonal proliferation of lymphocytes in response to lymphocyte-specific mitogens (Con A, PHA, and LPS response) is considered an *in vitro* correlate of the ability of lymphocytes to undergo clonal expansion in response to antigenic stimulation. Congenital hypothyroidism in *hyt/hyt* did not significantly affect the Con A and PHA response of splenocytes and, hence, the functional ability of T cells to proliferate. Although the Con A and PHA response of splenocytes was not significantly different in *hyt/hyt* compared with *hyt/+* mice, the ratio between the Con A and the PHA response (Con A to PHA ratio) was significantly decreased in hypothyroid mice (Table I). The differential responsiveness of murine T lymphocytes to PHA and Con A has, in the past, been used as a probe for T cell subsets (19). Thus, the altered Con A to PHA ratio in *hyt/hyt* mice observed here further supports the immunomodulatory effect of hypothyroidism on the normal balance among T cell populations. The LPS response of B cells was not different in *hyt/hyt* and *hyt/+* mice, which suggests that the proliferative phase during clonal expansion of B cells in response to antigen is not sensitive to hypothyroidism in *hyt/hyt* mice. Further studies are required to determine the consequences of these changes in peripheral T cell populations on T and B cell function in *hyt/hyt* mice.

The reduced absolute and adjusted spleen weight in hypothyroid compared with euthyroid mice are consistent with other reports on the sensitivity of the spleen to hypothyroidism (7, 9, 10) emphasizing the importance of normal thyroid function on spleen development. Support that the lower spleen weight associated with low-iodothyronine state might be due to changes in the immunological component of the spleen comes

from Fabris *et al.* (10), who attributed reduced spleen weight in *dw/dw* mice to a reduction in both size and number of lymphoid follicles, an underpopulation of the periateriolar sheath, and a reduction in cellularity of the red pulp. In *hyt/hyt* mice, it is not known whether the lower adjusted spleen weight is due to changes in proportions, amounts, or composition of white pulp and/or red pulp tissue. Beamer *et al.* (11) reported that the hypothyroid C.RF-*hyt* mouse (*hyt/hyt*) is slightly anemic. Anemia could result in lower red pulp matter of the spleen, thereby contributing to, or being solely responsible for, the reduced spleen weight observed in hypothyroid mice. Further studies are underway to examine the gross morphology of the spleen in euthyroid compared with hypothyroid C.RF-*hyt* mice.

Effects of Maternal Hypothyroidism. Body weight and visual appearance of 3- to 4-month-old progeny of hypothyroid dams were not different from that of euthyroid dams. It appears, therefore, that these aspects of development were not influenced by the thyroid state of the mother, or that the offspring's own thyroid function was compensating for the effects of maternal deficiencies during its fetal/neonatal life. However, long-term effects of maternal hypothyroidism on various aspects of immune development could be observed both in young-adult *hyt/+* and *hyt/hyt* offspring. Although the direction of maternal effects was similar in *hyt/hyt* and *hyt/+* offspring, maternal hypothyroidism had a greater influence on various aspects of immune development in *hyt/+* than in *hyt/hyt* progeny (data not shown). Long-term developmental effects of maternal hypothyroidism, noticeable in adult progeny, also have been reported for offspring with thyroidectomized mothers by Hendrich and Porterfield (20) and Porterfield and Hendrich (21). They observed lower body weights, lower liver weights (g), and higher circulating levels of thyroid-stimulating hormone (but normal thyroxine and triiodothyronine levels) in adult progeny of thyroidectomized rats (20, 21). Long-term effects of maternal hypothyroidism on immune development observed here include significantly greater absolute and adjusted thymus weight and thymocyte CD4 to CD8 ratios in progeny of hypothyroid *(hyt/hyt)* mothers compared with progeny of euthyroid mothers (Table II). To my knowledge, this is the first report describing larger thymuses and alterations in thymocyte subsets in progeny of hypothyroid mothers, suggesting long-term effects of maternal hypothyroidism on development and function of cell-mediated immunity in her offspring. Further studies are needed to determine the events that might have occurred between early embryonic development and weaning that influenced thymic growth and/or regression and T cell development into adulthood. The *hyt/hyt* mouse model, together with iodothyronine supplementation, offers an excellent opportunity to define the developmental stage during which normal maternal thyroid state is critical for the development of cell-mediated immunity in progeny.

Based on adjusted spleen weight data, it appears that maternal iodothyronine contribution *in utero* or during lactation is not required for the offspring to have normal spleen (secondary lymphoid organ) weight. However, further studies should be carried out to determine whether morphologic differences exist in spleens from mice with hypothyroid dams. Effects of maternal hypothyroidism on the function and proportions of T and B cell populations in the spleen are suggested by the lower mitogen responsiveness of splenocytes in progeny, especially in *hyt/+* offspring (data not shown), of hypothyroid dams (Table II). Interestingly, maternal hypothyroidism resulted in a greatly reduced LPS response in progeny (Table II), whereas congenital hypothyroidism was not associated with altered B cell proliferation (Table I) in response to LPS stimulation. Thus, it appears that components of the humoral immune response are more dependent on maternal than on an individual's own thyroid state. The marginally significant ($P = 0.069$) reduction in the Con A to PHA ratio, which was especially prominent in *hyt/+* offspring from hypothyroid mothers (data not shown), and the significant decrease in $CD4^+$ splenocytes and splenic CD4 to CD8 ratios (Table II) in progeny of hypothyroid dams compared with progeny of euthyroid dams, provide evidence for the necessity of normal maternal thyroid function to the development of a normal balance among T cell subsets in the spleen of offspring.

In the literature, evidence for placental and lactational transfer of iodothyronines is increasing (22). Placental transfer of iodothyronines can be bidirectional depending on the thyroid state of the fetus and the mother (21), and has been shown to exist at least by midgestation (23) and might already occur during early stages of embryogenesis (24). The study presented here also supports the concepts of maternal transfer of iodothyronine to her offspring. Furthermore, it was determined that subnormal thyroid function in *hyt/hyt* dams, presumably resulting in insufficient mother to fetus/neonate transfer of iodothyronines, can have long-term physiologic consequences on the immune system in offspring.

The mechanisms by which iodothyronines affect immune development and function have not been clearly established, and both direct effects (25–27) and indirect effects appear to be possible. Iodothyronines have been shown to affect the immune system indirectly by altering thymic hormone production and release (28, 29). Pituitary hormones, particularly adrenocorticotropic hormone, growth hormone and prolactin, and thyroid-stimulating hormone, also have been shown to be important for the normal development and function of the immune system (30–33). The *hyt/hyt* mice have

reduced numbers of somatotrophs and prolactin-producing cells, and increased numbers of "thyroidectomy cells" in the anterior pituitary gland (34). In addition, *hyt/hyt* mice were found to exhibit increased levels of hypothalamic thyrotropin-releasing hormone and circulating levels of thyroid-stimulating hormone, as well as depressed levels of hypothalamic corticotropin-releasing factor (34–36). Consequently, alterations of circulating levels of pituitary hormones due to low iodothyronine state might constitute another mechanism by which iodothyronines indirectly affect immune development and function.

This study revealed a number of alterations of immune development in hypothyroid mice and progeny of hypothyroid dams, and established the importance of normal thyroid function in the development and maintenance of the immune system. The effects of these alterations on immune function and other aspects of immune development need to be determined further to understand the interactions between the thyroid gland and the immune system. The C.RF-*hyt* mouse offers a unique system to study immune development and function in congenital and maternal hypothyroidism and, in conjunction with experimental manipulations of thyroid function and iodothyronine levels, offers an excellent opportunity to gain insight into the complex interactions between iodothyronines and the immune system.

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