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INVESTIGATIONS ON NATURALLY OCCURRING AND EXPERIMENTALLY INDUCED LYMPHOCYTIC THYROIDITIS IN DOGS

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INVESTIGATIONS ON NATURALLY OCCURRING AND EXPERIMENTALLY INDUCED LYMPHOCYTIC THYROIDITIS IN DOGS

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Sylvie Jacqueline Gosselin, D.V.M.

* * * * *

The Ohio State University
1980

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CHAPTER I

BIOCHEMICAL AND IMMUNOLOGICAL INVESTIGATIONS ON
HYPOTHYROIDISM IN DOGS

INTRODUCTION

Hypothyroidism resulting from destruction of the thyroid by lymphocytic thyroiditis or idiopathic follicular atrophy is an important metabolic disease of dogs. Most of the investigations in dogs with thyroiditis have been on colonies of laboratory beagles (2, 12, 21). Tucker (24) detected a 16.2% incidence of thyroiditis in young adult beagles with males and females being equally affected. Thyroiditis was diagnosed by careful histologic examination of the thyroid gland, since there were no clinical signs of hypothyroidism or macroscopic lesions. The focal lymphocytic thyroiditis observed in laboratory beagles usually was not associated with significant alterations in thyroid function; however, dogs with severe thyroiditis had a lower maximal thyroidal uptake and more rapid loss of $^{131}\text{I}$ compared to dogs with a mild or no thyroiditis (11).

Lymphocytic thyroiditis in dogs appears to represent an example of an autoimmune disease with a polygenic pattern of inheritance similar to that observed with chronic lymphocytic thyroiditis (Hashimoto's disease) in human beings (2,28). Mizejewski et al. (20) reported that thyroiditis in laboratory
beagles is similar serologically to human thyroiditis. Antibodies were present against thyroglobulin, a second colloid antigen, and a microsomal antigen. They were unable to find a positive correlation between the occurrence or height of the thyroglobulin antibody titers and the occurrence or severity of thyroiditis.

There is a paucity of data on circulating thyroid autoantibody levels and immune complexes compared to thyroid hormone values in pet dogs with spontaneous hypothyroidism. Therefore, the objectives of this study were: 1) to determine thyroid autoantibody titers in hypothyroidism and selected endocrine diseases of pet dogs; 2) to investigate the presence of circulating immune complexes in hypothyroid pet dogs and clinically normal pet dogs; and 3) to evaluate baseline thyroid hormone levels and response to thyrotropin (TSH) in dogs with hypothyroidism and other clinical endocrinopathies. In order to complete the latter objective it was necessary to investigate whether variations in the dose of TSH would influence the qualitative and quantitative patterns of response of canine thyroid gland to thyrotropin stimulation.

MATERIALS AND METHODS

Six clinically normal, purebred, male beagles from 1 to 3.5 years of age were given varying doses (0.1, 0.2, and 1.0 international units (I.U.)) of bovine thyrotropin (bTSH)\(^1\) per five pounds body weight intramuscularly separated by more than a one month interval in order to evaluate the dose-response to TSH. Blood samples were obtained before and at four, eight, 12 and 48 hours after

injection of bTSH. Serum concentrations of thyroxine (T₄) and triiodothyronine (T₃) were determined by radioimmunoassay.

**Experimental Dogs:** Blood samples from hypothyroid dogs were collected by venipuncture for assay of thyroid antibodies, circulating immune complexes and levels of T₃ and T₄ before and eight hours after bTSH stimulation. In this investigation, 1.0 I.U. bTSH/5 lbs body weight was used and an eight hour blood sample was collected in order to observe a consistent maximal response to stimulation. The tentative diagnosis of hypothyroidism in pet dogs was based on the history and clinical signs (18). The hypothyroid pet dogs were less active and alert, and usually had a gain in body weight without an associated increase in appetite. Dogs with hypothyroidism had difficulty in maintaining normal body temperature and were often "heat seekers". Changes in skin and hair coat were characterized by bilaterally symmetrical alopecia with an absence of pruritus, hyperkeratosis, local hyperpigmentation and in some myxedema. In order to determine the specificity of thyroid antibodies in dogs, serum was collected from 40 dogs with other spontaneous endocrinopathies or obesity presented to The Ohio State University Veterinary Teaching Hospital (ten dogs - hyperadrenocorticism; six dogs - hypoadrenocorticism; six dogs - hyperestrogenism; six dogs - acanthosis nigricans; six dogs - diabetes mellitus; six dogs - obesity) and compared to ten clinically normal adult pet dogs (five males, five females) and ten laboratory beagles (five males, five females). Serum was collected for T₃ and T₄ assay and a TSH-stimulation test (baseline and eight hours post-TSH) from all dogs of each group. Criteria for the diagnosis of these common endocrinopathies in the dog have been described elsewhere (18). The six dogs with acanthosis nigricans were all dachshunds, five males and one
female, with a mean age of 5.3 (range 2-14) years. The diagnosis was based upon clinical findings of lichenification of the skin involving predominately the axillary and inguinal areas with hyperkeratosis, hyperpigmentation and localized areas of alopecia, and normal laboratory data.

Preparation of Thyroid Antigens

Thyroid glands from dogs were collected immediately after euthanasia, frozen on dry ice, and stored at -20°C. The extraction of canine thyroglobulin was by a modification of the procedure described by Noble et al. (22). One hundred gm of thyroid glands were thawed and placed in a volume of phosphate-buffered saline (PBS) (pH = 7.2) equal in ml to twice the weight of the tissue in grams. The thyroid tissue was ground in a Waring blender for one minute. Flasks containing the homogenate were placed overnight on a horizontal action shaker. Subsequently, the thyroid homogenate was centrifuged at 20,000 rpm for two hours in a Beckman L2-65B ultracentrifuge with a type 21 rotor. The supernatant was collected free of dense pellet below and the lipid layer above. The protein concentration was determined by the biuret reaction and adjusted to 30 mg/ml. This crude extract of canine thyroid glands was stored at -20°C.

Canine thyroglobulin was isolated from the thyroid extract by passing ten ml of the extract through a Sephadex G-200 column and the column was washed with PBS. Fractions were collected automatically at six minute intervals. Thyroglobulin was recovered in the first protein peak eluting from the column. The column-purified thyroglobulin was adjusted to a concentration of 1 mg/ml and was stored at -20°C.
Chromic Chloride Passive Hemagglutination (CCH) Test  The thyroglobulin autoantibodies were evaluated by the CCH test. The CCH test was a modification of the procedure by Poston (23). Sheep red blood cells (S-RBC) were washed three times with isotonic saline. To coat the S-RBC with thyroglobulin, 0.5 ml of 0.27 M piperazine buffer was added to a 40 ml centrifuge tube. The 0.1 ml of canine thyroglobulin (0.5 mg/ml), 0.1 ml of packed washed erythrocytes, and 0.2 ml of $5 \times 10^{-3} M$ chromic chloride ($\text{CrCl}_3$) were added sequentially to the tube. The suspension was agitated gently for four to five minutes. The reaction was stopped by the addition of 30 ml of saline and the cells were washed three times in saline by centrifuging at 1250-1350 rpm for eight minutes. Sheep red blood cells were used at a cell concentration of 0.66%. The test was performed with microtiter "V" bottom plates.

Tanned Cell Hemagglutination (TCH) Test  The TCH test was performed according to the method described by Weir (30) to determine the level of circulating thyroglobulin antibodies in dogs. Sheep red blood cells were washed three times with PBS. An equal volume of freshly diluted tannic acid (1:30000) in PBS was added to a 2.5% S-RBC solution and incubated for ten minutes at 37°C. The S-RBC solution was washed three times with PBS and resuspended in 40 ml PBS. Three solutions were prepared, each containing 1.0 ml of tanned cells and 4.0 ml of PBS. One ml of purified canine thyroglobulin (0.5 mg/ml) was added to the first solution, 1.0 ml of PBS was added to the second solution, and 1.0 ml of bovine albumin was added to the third solution. The solutions were incubated at room temperature for 30 minutes, washed two times with 2.0 ml of normal rabbit serum in PBS (1:30), and resuspended in 1.0 ml of normal
rabbit serum in PBS (1:30). The tanned cells were added to serial dilutions of the test serum in "V" bottom microplates.

**Complement Fixation (CF) Test** Thyroid microsomal antigen was determined by the complement fixation micromethod of Mayer (19). The canine thyroid extract was frozen at -20°C until use. The CF test system utilized 2.8% S-RBC washed three times in veronal buffer and sensitized with three (minimum hemolytic dose) units of hemolysin and four or five units of 50% hemolytic units of complement. All dog sera were titrated with a 1:6 dilution of canine thyroid extract antigen as determined from a prior titration with a standard serum. The test was performed in microtiter "U" bottom plates.

**Mastocytoma Cell (MC) Assay** Immune complexes present in the circulation were evaluated by the MC test (25). This test is based on the binding of soluble immune complexes (composed of IgG antibodies and their corresponding antigens) to the Fc receptor on MC (P-815) (7) propagated in the peritoneal cavity of DBA/2 mice. The binding was determined by the inhibition of rosette formation with S-RBC sensitized with a subagglutinating dose of the IgG fraction of rabbit anti-S-RBC serum. Sera obtained from 25 dogs with hypothyroidism and ten clinically normal pet dogs were heat inactivated and tested at dilutions of 1:10, 1:20, 1:40, 1:80 and 1:160. Serum from a germfree dog and PBS served as controls. The various dilutions of serum were mixed with an equal volume of MC (5 X 10^6 cells/ml) in a "U" bottom microplate and

---

2 Veronal buffer - Sigma Chemical Co., St. Louis, Missouri.
3 MC (P815) were kindly supplied by Dr. T. Flanagan, Department of Microbiology, SUNY, Buffalo, New York.
4 Cordis Laboratories, Miami, Florida.
incubated at 4°C for one hour. Subsequently, 50 µl of sensitized S-RBC at a concentration of 0.5% v/v was added to the cultures, centrifuged for five minutes at 500 rpm, and incubated at 4°C for one hour. The pelleted cells were then gently resuspended. The number of rosette forming cells per 100 cells was determined and expressed in percent. A difference exceeding ten percent between the number of rosette forming cells in the serum of hypothyroid and the controls was considered to indicate the presence of circulating immune complexes.

**Hormone Assays** Serum thyroxine ($T_4$) and triiodothyronine ($T_3$) for the baseline and eight hours after TSH-stimulation were determined by radioimmunoassay (V.K. GanJam, unpublished data). Changes in serum $T_3$ and $T_4$ at different intervals following injection of 0.1, 0.2 and 1.0 I.U. bTSH were evaluated by two-way analysis of variance. Differences between the three dose levels of TSH were determined by Dunnett's t-test. Differences in blood $T_3$ and $T_4$ between the hypothyroid group and the other groups of dogs at baseline and eight hours after TSH stimulation were evaluated by a priori contrast (Table I and II). Differences between clinically normal dogs and dogs with other endocrinopathies or obesity at baseline and eight hours after TSH stimulation were evaluated by a priori contrast and the Newman-Keuls test (Table II). Baseline and eight hour post-TSH values for serum $T_3$ and $T_4$ in individual groups were compared by the Student t-test.
RESULTS

The CCH test revealed that 12 of 25 pet dogs (48%) with hypothyroidism had serum antibodies (1:20 to 1:2560) against thyroglobulin (Table I). The highest titer of thyroglobulin antibodies (1:2560) was in the only dog with a positive microsomal antibody titer as determined by the complement fixation test. By comparison the TCH test revealed thyroid autoantibody titers (1:40 to 1:160) in only six of the same 25 dogs (24%) with hypothyroidism. The titers were consistently lower than detected by the CCH test. The CCH test was more sensitive than the TCH test, used less antigen, and the solution of thyroglobulin-coated S-RBC could be kept for three weeks without affecting the sensitivity of the test.

Thyroid autoantibody titers were evaluated in selected clinical endocrinopathies of pet dogs and clinically normal dogs to determine the specificity of alterations in thyroglobulin or microsomal antibodies. Four dogs with acanthosis nigricans had antibodies against thyroglobulin with a CCH titer of 1:20. All dogs with acanthosis nigricans had normal or elevated baseline thyroid hormone levels and a similar response to TSH stimulation compared to clinically normal pet dogs (Table II). One of six dogs with hyperestrogenism (CCH titer 1:40) and two of ten clinically normal laboratory beagles (male-CCH titer 1:320, TCH titer 1:20; female-CCH titer 1:20) had antibodies against thyroglobulin. All dogs evaluated with hypoadrenocorticism, hyperadrenocorticism (cortisol-excess), diabetes mellitus, obesity, and clinically normal pet dogs did not have circulating thyroglobulin or microsomal antibodies detectable by the CCH, TCH and CF tests (Table II).
Circulating immune complexes determined by the MC-assay were present in five of 25 dogs with hypothyroidism at a serum dilution of 1:10 (Table I). Only one hypothyroid dog with circulating immune complexes also had thyroglobulin autoantibodies (1:80) in the serum. Circulating immune complexes were absent in the serum of ten clinically normal pet dogs.

Clinically normal beagles administered 0.1, 0.2, and 1.0 I.U. bTSH/5 lbs body weight had significantly elevated serum 

\[ T_3 \] and \[ T_4 \] levels within four hours. Peak increases for serum \[ T_3 \] and \[ T_4 \] were observed either at eight hours (0.1 and 0.2 I.U. bTSH) or 12 hours (1.0 I.U. bTSH). The response of serum \[ T_3 \] to all three doses of bTSH was similar except at 12 and 24 hours when the increase was significantly greater with 1.0 I.U. bTSH. The response of serum \[ T_4 \] to 0.1 and 0.2 I.U. bTSH was similar except at 48 hours. The increase in serum \[ T_4 \] after 1.0 I.U. bTSH was significantly different from the two lower dose levels at four, eight, 12 and 24 hours. Serum \[ T_3 \] and \[ T_4 \] were decreasing at 12 hours after receiving 0.1 and 0.2 I.U. of bTSH, reaching baseline values or lower at 24 to 48 hours. Thyroid hormone levels continued to increase between eight and 12 hours after 1.0 I.U. of bTSH but had decreased at 24 hours. The percent increase above baseline values was used to illustrate the effect of the three doses of bTSH on circulating \[ T_3 \] and \[ T_4 \] levels in clinically normal laboratory beagles (Fig. 1). The maximal percent increase in serum \[ T_4 \] above baseline at eight hours was significantly different between 0.1 or 0.2 and 1.0 I.U. of bTSH but not between 0.1 and 0.2 I.U. of bTSH. The maximal percent increase in serum \[ T_3 \] was different only between 0.1 and 1.0 I.U. of bTSH.

The circulating levels of thyroid hormones did not increase significantly eight hours after injection of 1.0 I.U. bTSH/5 lbs body weight (\[ T_3 \] = 38.5 \pm 4.0
ng/dl; \( T_4 = 0.84 \pm 0.06 \mu g/dl \) in dogs with hypothyroidism (Table I). Dogs with spontaneous hypothyroidism had a significantly decreased \((p<0.001)\) response for serum \( T_3 \) and \( T_4 \) at baseline and eight hours post-TSH compared to clinically normal pet dogs and laboratory beagles (Table I and Table II).

The increase in circulating thyroid hormone levels at eight hours post-TSH (1.0 I.U. bTSH/5 lbs) was significant in clinically normal pet dogs \((p<0.001)\), laboratory beagles \((p<0.001)\), and pet dogs with other endocrinopathies or obesity (Table II). Significant differences were not found in the response to TSH between males and females in normal pet dogs and laboratory beagles. However, serum \( T_3 \) and \( T_4 \) at baseline in beagles were higher compared to normal dogs and the increase in \( T_4 \) was considerably greater in beagles following TSH stimulation (Table II). The response of serum \( T_3 \) levels in hypothyroid dogs to TSH stimulation was significantly decreased \((p<0.01)\) compared to dogs with hyperadrenocorticism, hypoadrenocorticism, diabetes mellitus, acanthosis nigricans, hyperestrogenism or obesity. The response of serum \( T_4 \) levels in hypothyroid dogs to TSH stimulation also was significantly decreased \((p<0.05)\) compared to dogs with hyperadrenocorticism, acanthosis nigricans, hyperestrogenism or obesity (Tables I and II).

Dogs with cortisol-excess had a significantly depressed response of serum \( T_4 \) at eight hours post-TSH stimulation compared to clinically normal pet dogs (Table II). The response to TSH stimulation for \( T_3 \) and \( T_4 \) also was depressed in dogs with hypoadrenocorticism, and diabetes mellitus. The baseline serum \( T_3 \) and \( T_4 \) levels were significantly elevated in dogs with acanthosis nigricans and in obese dogs but the response to TSH was similar as in normal pet dogs.
DISCUSSION

The results of this investigation demonstrated circulating antibody titers against thyroglobulin in 48% of pet dogs with hypothyroidism by the chromic chloride passive hemagglutination test but only four percent had positive titers against thyroid microsomal antigens. Hypothyroidism in the dog usually is the result of a primary disease of the thyroid gland, especially idiopathic follicular atrophy and lymphocytic thyroiditis (14). There are no specific clinical signs that distinguish hypothyroidism caused by idiopathic follicular atrophy from lymphocytic thyroiditis. The presence of circulating thyroglobulin autoantibodies in pet dogs with hypothyroidism would suggest that a thyroid lesion of lymphocytic thyroiditis was responsible for the disease. There are four thyroid antigens against which specific antibodies may be produced in lymphocytic "autoimmune" thyroiditis (Hashimoto's disease) i.e., 1) thyroglobulin, 2) microsomal cytoplasmic antigen (a lipoprotein in the membrane of the small vesicles containing newly synthesized thyroglobulin emerging from the Golgi apparatus), 3) second antigen (CA-2) of the colloid (a protein containing no iodine), and 4) cell-surface antigen (9).

A similar frequency of positive thyroglobulin autoantibody titers has been reported in laboratory beagles with lymphocytic thyroiditis as in pet dogs but in addition they had a much higher incidence of circulating antibodies against thyroid microsomal antigen (20). The relatively low titers of thyroglobulin autoantibodies detected in pet dogs (1:20 to 1:2560) were in contrast to the findings often reported in human patients with chronic lymphocytic thyroiditis (Hashimoto's disease) and hypothyroidism where there usually were high titers
of both microsomal and thyroglobulin antibodies (10). However, the later stages of the atrophic form of lymphocytic thyroiditis often is associated with hypothyroidism, low titer or absence of thyroglobulin antibodies, and an absence of microsomal antibodies (13). Lymphocytic thyroiditis with clinical hypothyroidism in pet dogs often is seen in the later stage of the disease with widespread destruction of the thyroid (14). The reason for the lower titers of thyroglobulin antibodies may be related to binding of antibodies with antigen in the thyroid gland as has been demonstrated in experimentally induced thyroiditis of mice (6).

Antibodies against thyroglobulin may be present in patients with thyroid carcinoma and autoimmune diseases such as pernicious anemia, systemic lupus erythematosus, and idiopathic atrophy of the adrenal in human beings (8). In our investigation there appeared to be an association between low circulating levels of thyroglobulin antibodies and acanthosis nigricans. Although the etiology of acanthosis nigricans in dogs is uncertain an autoimmune mechanism has not been eliminated as being involved in the pathogenesis of the disease.

It has been suggested that in the pathogenesis of autoimmune thyroiditis a specific thyroid antigen, unknown to lymphocytes, is released and sensitizes lymphocytes. This mechanism has been described previously as the secluded antigen theory and was favored as a possible explanation for the etiology of lymphocytic thyroiditis or Hashimoto's disease in man. However, the antigen (thyroglobulin) most suspected of playing an etiologic role in autoimmune thyroiditis has been shown to leak from its secluded site in the lumen of thyroid follicles into the circulation in patients with non-toxic goiter and after radioiodine therapy without inducing thyroiditis (9). In addition, there is
thyroglobulin leakage and the presence of thyroglobulin-binding B-lymphocytes in all individuals before birth (27). Furthermore, it has not been possible to demonstrate immunologic differences between the thyroglobulin from individuals with Hashimoto's thyroiditis and normal thyroid glands (29). There is accumulating evidence that thyroglobulin antibodies recognize the thyroid antigens and facilitate the phagocytic role of killer cells. This cooperative role of thyroglobulin antibodies with the killer cells may be important in the pathogenesis of the thyroid lesions present in autoimmune thyroiditis (1).

Circulating immune complexes evaluated by the MC microassay were found in 20% of dogs with hypothyroidism. A similar incidence of circulating immune complexes has been detected in human patients with various autoimmune and nonautoimmune mediated thyroid diseases (4). A recent study detected the presence of soluble thyroglobulin-anti-thyroglobulin immune complexes (Tg-IC) (5.5%) and other immune complexes unrelated to thyroglobulin (16.7%) in the sera of patients with chronic lymphocytic thyroiditis (Hashimoto's disease) (17). Indirect evidence of circulating thyroid immune complexes has been provided by infrequent reports of thyroglobulin and microsomal antigens associated with IgG and complement, as determined by immunofluorescence in glomeruli of human patients with thyroid autoimmune disorders and glomerulonephritis (16). Therefore, circulating Tg-IC may be only a fraction of the total immune complexes found in thyroid disorders and the nature of other immune complexes remains to be established. Further studies will be required to determine whether these circulating immune complexes play a pathogenic role in thyroid autoimmune diseases.

Different doses of bovine thyrotropin were used to test whether variations in dose would influence the qualitative and quantitative patterns of response of
the canine thyroid gland to stimulation as reflected by changes in circulating serum $T_3$ and $T_4$ levels. A similar qualitative pattern of response for serum $T_3$ was observed regardless of the dose of TSH; however, the pattern of response of serum $T_4$ was different between 0.1 or 0.2 and 1.0 I.U. TSH. The striking increase at four, eight, 12 and 24 hours after 1.0 I.U. TSH was significantly greater than with the two lower doses. The peak increase for serum $T_4$ and $T_3$ were observed either at eight (0.1 and 0.2 I.U.) or 12 (1.0 I.U.) hours post-TSH. A similar type of dose-response to TSH has been reported in normal human beings (26). Belshaw and Rijnberk (3) found the peak increase in serum $T_4$ following bTSH (10 I.U.) stimulation to be at eight hours in 80% of dogs with a small additional increase at 12 hours in the remaining dogs.

Pet dogs with naturally occurring hypothyroidism had a significantly lower serum $T_3$ and $T_4$ at baseline and eight hours post-TSH when compared to clinically normal pet dogs and laboratory beagles. Similar differences have been reported recently by Belshaw and Rijnberk (3) in a large series of dogs with hypothyroidism using a sensitive radioimmunoassay. Clinical hypothyroidism in pet dogs usually is a result of widespread destruction of the thyroid gland by lymphocytic thyroiditis or idiopathic follicular atrophy (14), thereby resulting in an inability of follicular cells to respond to thyrotropin stimulation by an increased release of thyroid hormone. Quantitation of circulating levels of $T_3$ and $T_4$ before and after thyroid challenge by thyrotropin was useful in the separation between certain normal and hypothyroid dogs. Hypothyroid pet dogs with circulating thyroid hormone levels in the low normal range were unable to significantly increase serum $T_3$ and $T_4$ levels after eight hours in response to TSH stimulation as clinically normal pet dogs. An unexpected finding was the
significantly greater increase in serum thyroxine in response to thyrotropin stimulation in laboratory beagles compared to normal pet dogs.

The dogs evaluated with the syndrome of cortisol-excess had a significantly depressed response of T₄ eight hours post-TSH. Baseline values for T₃ and T₄ were lower in dogs with cortisol-excess, as has been observed by other investigators (3), possibly due to decreased hormonal binding to plasma proteins, but these differences were not significant. The response to thyrotropin also was depressed in dogs with hypoadrenocorticism, and diabetes mellitus compared to normal pet dogs (Table II). Male dogs with functional disturbances and lesions of hyperestrogenism due to a Sertoli cell tumor of the testis did not have elevated levels of thyroid hormone (Table II). Pregnancy in women often is associated with an increase in circulating thyroid hormone levels due to estrogen-induced synthesis of thyroxine-binding protein by the liver (5).

**SUMMARY**

Circulating antibody titers (1:20 to 1:2560) against thyroglobulin were demonstrated in 48% of pet dogs with hypothyroidism by the chromic chloride passive hemagglutination test. Four of six dogs with acanthosis nigricans (1:20) and one of six male dogs with hyperestrogenism (1:40) had low titers of antibody against thyroglobulin whereas clinically normal pet dogs and dogs with other selected endocrinopathies (hypoadrenocorticism, cortisol-excess, diabetes mellitus) or obesity were consistently negative. Circulating immune complexes evaluated by the mastocytoma cell-assay were present in the sera of 20% of pet dogs with hypothyroidism but were absent in clinically normal dogs.
Although variations in dose significantly altered the quantitative response of the thyroid gland to TSH the qualitative pattern of response was similar for T₃ but not T₄ in clinically normal laboratory beagles. The peak increases for serum triiodothyronine and thyroxine were observed either at eight (0.1 and 0.2 I.U. bTSH/5 lbs) or 12 (1.0 I.U. bTSH/5 lbs) hours post-TSH.

Dogs with naturally occurring hypothyroidism had a decreased serum T₃ and T₄ at baseline and eight hours post-TSH (1.0 I.U. bTSH/5 lbs) compared to clinically normal pet dogs, laboratory beagles and dogs with other clinical endocrinopathies. The consistent lack of a significant increase of serum T₃ and T₄ in response to TSH was necessary for the separation of certain hypothyroid from euthyroid pet dogs in which the baseline level of thyroid hormones were equivocal.
Figure 1.1. Serum thyroxine (left) and triiodothyronine (right) expressed as percent increase above baseline at 4, 8, 12, 24 and 48 hours following the intramuscular (IM) injection of 0.1, 0.2, and 1.0 international units (u) TSH. Data based on six adult male beagle dogs per dose level of TSH. (Mean ± standard error).
SERUM THYROXINE (BY RADIOIMMUNOASSAY) (PERCENT INCREASE ABOVE BASELINE)

SERUM TRIIODOTHYRONINE

HOURS POST-TSH INJECTION (1M)

TSH (units/mL)

0.1 u
0.2 u
0.5 u
1.0 u

Fig. 1
Table 1.1. Serum Thyroxine ($T_4$) and Triiodothyronine ($T_3$) Before and 8 Hours After TSH Injection (1.0 I.U. bTSH/5 lbs), Thyroid Autoantibody Titers and Circulating Immune Complexes in 25 Pet Dogs with Hypothyroidism.

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>$T_3$ Baseline (ng/dl)</th>
<th>$T_4$ Baseline (ug/dl)</th>
<th>$T_3$ 8 hr Post-TSH (ng/dl)</th>
<th>$T_4$ 8 hr Post-TSH (ug/dl)</th>
<th>CCH $^d$</th>
<th>TCH$^e$</th>
<th>CF $^f$</th>
<th>Serum Dilution 1:10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.2</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
<td>1:20</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>34.1</td>
<td>0.4</td>
<td>30.0</td>
<td>0.4</td>
<td>1:80</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>70.2</td>
<td>0.6</td>
<td>73.9</td>
<td>0.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>30.0</td>
<td>0.6</td>
<td>30.0</td>
<td>0.7</td>
<td>1:80</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>18.2</td>
<td>1.2</td>
<td>21.5</td>
<td>1.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>43.4</td>
<td>1.1</td>
<td>51.5</td>
<td>0.8</td>
<td>1:2560</td>
<td>1:80</td>
<td>1:32</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
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<td>1.5</td>
<td>52.9</td>
<td>1.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>34.3</td>
<td>1.1</td>
<td>52.4</td>
<td>1.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
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<td>0.9</td>
<td>84.9</td>
<td>0.8</td>
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<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>40.3</td>
<td>0.6</td>
<td>42.8</td>
<td>0.7</td>
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<td>1:40</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>59.8</td>
<td>0.8</td>
<td>63.4</td>
<td>0.7</td>
<td>1:160</td>
<td>1:40</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>65.0</td>
<td>0.6</td>
<td>-</td>
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<td>0</td>
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<td>-</td>
</tr>
<tr>
<td>14</td>
<td>48.2</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>50.8</td>
<td>1.2</td>
<td>38.0</td>
<td>1.1</td>
<td>1:40</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>28.4</td>
<td>1.1</td>
<td>41.2</td>
<td>1.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>12.2</td>
<td>0.9</td>
<td>16.7</td>
<td>1.0</td>
<td>1:640</td>
<td>1:160</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>40.3</td>
<td>0.6</td>
<td>42.8</td>
<td>0.7</td>
<td>1:80</td>
<td>1:40</td>
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</tr>
<tr>
<td>19</td>
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<td>5.1</td>
<td>0.9</td>
<td>1:640</td>
<td>1:80</td>
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<td>-</td>
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<tr>
<td>20</td>
<td>48.6</td>
<td>0.7</td>
<td>45.4</td>
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<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>61.5</td>
<td>1.6</td>
<td>51.7</td>
<td>1.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>5.0</td>
<td>0.9</td>
<td>5.0</td>
<td>0.8</td>
<td>1:40</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>30.9</td>
<td>0.3</td>
<td>25.5</td>
<td>0.3</td>
<td>1:20</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>15.3</td>
<td>0.3</td>
<td>22.1</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>7.1</td>
<td>0.2</td>
<td>11.9</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>38.7 ± 4.1 $^b$</td>
<td>0.80 ± 0.07 $^b$</td>
<td>38.5 ± 4.0 $^{ab}$</td>
<td>0.84 ± 0.06 $^{ab}$</td>
<td>12/25 $^e$</td>
<td>6/25 $^e$</td>
<td>1/25 $^e$</td>
<td>3/25 $^e$</td>
</tr>
</tbody>
</table>

Notes:
- $^a$ Statistically significant compared to baseline.
- $^b$ Data include normal saline controls (N.S.).
Table 1.1. (Continued)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Significantly different from clinically normal pet dogs at p&lt;0.05 comparing individual mean values at baseline and 8 hours post-TSH by a priori contrasts.</td>
</tr>
<tr>
<td>b</td>
<td>Mean ± Standard Error</td>
</tr>
<tr>
<td>c</td>
<td>Dogs with Positive Antibody Titer or Immune Complexes/Total Number of Dogs</td>
</tr>
<tr>
<td>d</td>
<td>CCH: Chromic Chloride Hemagglutination Test</td>
</tr>
<tr>
<td>e</td>
<td>TCH: Tanned Red Cell Hemagglutination Test</td>
</tr>
<tr>
<td>f</td>
<td>CF: Complement Fixation Test</td>
</tr>
</tbody>
</table>

(N.S.) Not Significant. Comparison Between Baseline and 8 hr post-TSH by the Student's t-test.
Table 1.2. Serum Thyroxine (T\textsubscript{4}) and Triiodothyronine (T\textsubscript{3}) Before and 8 hours After TSH Injection (1.0 I.U. bTSH/5 lbs.) Compared With Positive Thyroid Autoantibody Titers in Selected Clinical Endocrinopathies or Obesity in Pet Dogs and Clinically Normal Dogs.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Dogs</th>
<th>T\textsubscript{3} Baseline (ng/dl)</th>
<th>T\textsubscript{4} Baseline (ug/dl)</th>
<th>T\textsubscript{3} 8 hrs post-TSH (ng/dl) (p&lt;0.001)</th>
<th>T\textsubscript{4} 8 hrs post-TSH (mg/dl) (p&lt;0.001)</th>
<th>CCH\textsuperscript{c}</th>
<th>TCH\textsuperscript{d}</th>
<th>CF\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperadrenocorticism</td>
<td>10</td>
<td>105.2±10.1\textsuperscript{a}</td>
<td>1.48±0.19\textsuperscript{a}</td>
<td>202.6±23.0\textsuperscript{a} (p&lt;0.001)</td>
<td>3.07±0.28\textsuperscript{ab} (p&lt;0.001)</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10</td>
</tr>
<tr>
<td>Hypoadrenocorticism</td>
<td>6</td>
<td>74.6±9.6\textsuperscript{a}</td>
<td>1.71±0.11\textsuperscript{a}</td>
<td>120.2±14.8\textsuperscript{ab} (p&lt;0.01)</td>
<td>2.4±0.13\textsuperscript{b} (p&lt;0.01)</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>6</td>
<td>95.8±14.8\textsuperscript{a}</td>
<td>1.91±0.20\textsuperscript{a}</td>
<td>135.3±11.6\textsuperscript{ab} (p&lt;0.01)</td>
<td>2.95±0.41\textsuperscript{b} (p&lt;0.05)</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Acanthosis Nigricans</td>
<td>6</td>
<td>121.4±20.2\textsuperscript{ab}</td>
<td>2.56±0.24\textsuperscript{ab}</td>
<td>214.2±25.2\textsuperscript{ab} (p&lt;0.01)</td>
<td>6.35±1.58\textsuperscript{a} (p&lt;0.05)</td>
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<td>Hyperestrogenism</td>
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<td>80.1±15.5\textsuperscript{a}</td>
<td>1.80±0.24\textsuperscript{a}</td>
<td>189.3±21.6\textsuperscript{ab} (p&lt;0.01)</td>
<td>3.79±0.31\textsuperscript{a} (p&lt;0.01)</td>
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<td>0/6</td>
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<td>160.2±32.5\textsuperscript{ab}</td>
<td>3.33±0.35\textsuperscript{ab}</td>
<td>256.5±44.0\textsuperscript{ab} (p&lt;0.01)</td>
<td>6.25±0.93\textsuperscript{ab} (p&lt;0.01)</td>
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<td>Clinically Normal: Pet Dogs</td>
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<td>1.94±0.08\textsuperscript{a}</td>
<td>220.9±13.9\textsuperscript{ab} (p&lt;0.001)</td>
<td>5.91±0.81\textsuperscript{ab} (p&lt;0.001)</td>
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<td>0/5</td>
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<td>228.3±18.8</td>
<td>6.5±1.2</td>
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<td>0/5</td>
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<td>5.3±1.1</td>
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<td>Laboratory Beagles</td>
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<td>13.48±2.41\textsuperscript{ab} (p&lt;0.001)</td>
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Table 1.2. (Continued)

Mean ± Standard Error

- **a**: Significantly different from hypothyroid dogs at p<0.05 comparing mean values at baseline and 8 hours post-TSH.
- **b**: Significantly different from clinically normal pet dogs at p<0.05 comparing mean values at baseline and 8 hours post-TSH.

- **c**: CCH: Chronic Chloride Hemagglutination Test
- **d**: TCH: Tanned Red Cell Hemagglutination Test
- **e**: CF: Complement Fixation Test
CHAPTER II

HISTOPATHOLOGIC AND ULTRASTRUCTURAL EVALUATION OF THYROID LESIONS ASSOCIATED WITH HYPOTHYROIDISM IN DOGS

INTRODUCTION

Hypothyroidism in pet dogs is a metabolic disorder which can result from primary diseases of the thyroid gland or be secondary to long-standing pituitary or hypothalamic lesions that interfere with the release of thyrotropin (TSH) or thyrotropin-releasing hormone. Hypothyroid dogs have a gradual decline in vigor and physical activity, and are often "heat seekers" (12). Varying degrees of alopecia and epidermal atrophy were reported in 48% of hypothyroid dogs (17). Hypothyroidism in pet dogs was associated with a low level of serum triiodothyronine (38.7 ± 4.1 ng/dl) and thyroxine (0.8 ± 0.07 μg/dl) and circulating thyroid hormone levels did not increase significantly after TSH stimulation (7). These changes in thyroid function were specific for hypothyroid dogs when compared to other dogs with selected endocrinopathies or normal pet dogs. Thyroglobulin autoantibodies were demonstrated in 48% of pet dogs with hypothyroidism and may be related to the pathogenesis of thyroiditis (7). Laboratory beagles with naturally occurring lymphocytic thyroiditis also have circulating thyroid autoantibodies (14) but the focal thyroiditis was not associated with clinical signs of hypothyroidism (19). The specific objective of
this study was to investigate by light and electron microscopy thyroid lesions resulting in clinical hypothyroidism of pet dogs.

MATERIALS AND METHODS

Thyroid glands from 16 dogs with clinical hypothyroidism were available for evaluation by light and electron microscopy. The tentative diagnosis of hypothyroidism in pet dogs was based upon the history and clinical signs (12). Hypothyroid dogs (6 males, 10 females) ranged from 3 to 15 years of age (mean: 7.8 years) and had a clinical history of gain in body weight with normal food consumption, were less active and alert, sought warm places in their environment, and often developed patchy alopecia. The serum thyroxine level either assayed by column chromatography (range: 0.1 to 0.6 µg/dl; mean: 0.3 µg/dl) or radioimmunoassay (range: 0.5 to 2.4 µg/dl; mean: 0.9 µg/dl) was low or in the low normal range with a lack of response to TSH stimulation (1.0 I.U. bovine TSH/5 lb body weight). The serum cholesterol level was elevated (range: 252 to 2300 mg/dl; mean: 781.6 mg/dl) in hypothyroid pet dogs.

Thyroid glands were collected during surgery or immediately after killing at necropsy. A section of thyroid gland was fixed in Bouin's fluid, cut at 4µ, and stained with either hematoxylin and eosin or periodic acid-Schiff (PAS). Ten 1.0 mm cubes from several areas of the thyroid gland were collected for electron microscopy, fixed in ice cold 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, and post-fixed with 1.33% osmium tetroxide in s-collidine at pH 7.2. Tissue blocks were dehydrated in ascending concentrations of ethanol, transferred to propylene oxide, and embedded in Epon 812. Thin sections were cut at
and stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope.

**RESULTS**

Two different thyroid lesions were associated with clinical hypothyroidism in pet dogs. Thyroid glands with lymphocytic thyroiditis were either slightly enlarged (1 dog) or atrophic (6 dogs) and thyroids with idiopathic follicular atrophy were normal (1 dog) or smaller than normal (8 dogs).

Histopathologic alterations in the thyroid glands of 7 dogs with lymphocytic thyroiditis consisted of a multifocal to diffuse infiltration by lymphocytes, sometimes with the formation of lymphoid nodules, plasma cells and macrophages (fig. 1). The remaining thyroid follicles were small and lined by columnar epithelial cells. Lymphocytes, macrophages, and degenerate follicular cells were present in the vacuolated colloid within the thyroid follicles. Thyroid C-(parafollicular) cells were observed in small nests or nodules and often appeared more prominent than in normal dogs. In dogs with lymphocytic thyroiditis, some remaining follicular cells were transformed into large oxyphilic cells with a densely eosinophilic granular cytoplasm.

The thyroid lesion of lymphocytic thyroiditis appeared to progress to an end-stage form where the majority of the thyroid gland was replaced by mature fibrous connective tissue with few scattered foci of inflammatory cells. Thyroid follicles were sparse and widely separated, and contained only a small amount of vacuolated colloid (fig. 2). Thyroid nodular hyperplasia (1 dog) and atherosclerotic changes of the small branches of the cranial thyroid artery (1 dog) were seen occasionally in dogs with thyroiditis.
Lymphocytic thyroiditis was characterized ultrastructurally by the presence of numerous lymphocytes, plasma cells, and macrophages in the interstitium, migrating through the follicular wall, and in the lumen admixed with colloid and degenerate follicular cells (fig. 3). Inflammatory cells also were numerous in lumens of interfollicular capillaries. The basement membrane around thyroid follicles either was densely thickened (between 3.5 and 12.0 μm in thickness) (4 dogs) (fig. 4) or discrete electron-dense deposits were present between the follicular cells and the basement membrane (3 dogs) (fig. 5).

The hypertrophied cells lining small thyroid follicles had a large cytoplasmic area with dilated profiles of rough endoplasmic reticulum and a well developed Golgi apparatus. Long microvilli projected into the follicular lumen that often were compressed by the numerous inflammatory cells and degenerate follicular cells present in the colloid. Many follicular cells had small intracytoplasmic microfollicles which were filled with a finely granular electron-dense material similar to colloid and had long microvilli (fig. 4). Scattered oxyphilic cells were present in thyroids of 3 dogs that had numerous mitochondria in their cytoplasm but a sparse development of rough endoplasmic reticulum and a small Golgi apparatus (fig. 6). The remaining follicular cells in lymphocytic thyroiditis were in different stages of degeneration and contained occasional cytosegresomes, dilated profiles of rough endoplasmic reticulum, and swollen mitochondria with myelin bodies. The thyroid C-cells ultrastructurally were normal.

The end-stage form of lymphocytic thyroiditis was characterized by a marked increase in collagen fibers surrounding small follicles or groups of follicular cells (fig. 7). These remaining hypertrophied follicular cells had
dilated profiles of rough endoplasmic reticulum, a prominent Golgi apparatus, and occasional intracytoplasmic microfollicles.

The thyroid lesion in the remaining 9 dogs with clinical hypothyroidism was interpreted as idiopathic follicular atrophy. The overall gland size was decreased, thyroid follicles were reduced in number and replaced by adipose connective tissue but there was minimal evidence of infiltration by inflammatory cells. The early lesion of follicular atrophy was observed in the youngest dog (3 years old) with clinical signs of hypothyroidism but with baseline thyroid hormone levels determined by radioimmunoassay (2.4 μg/dl) and response to TSH stimulation (5.2 μg/dl at 8 hour) in the normal range. One part of the thyroid gland contained small follicles lined by tall columnar follicular cells often with little colloid. Immediately adjacent thyroid follicles were normal (fig. 8). Individual or small groups of degenerate follicular cells with eosinophilic cytoplasm and pyknotic nuclei were present in the follicular wall, colloid, and interstitium (fig. 9).

In dogs with clinical hypothyroidism and low circulating levels of thyroid hormones, the thyroid gland was composed predominantly of adipose connective tissue with only occasional clusters of small follicles containing a vacuolated colloid (fig. 10). When the thyroid was reduced markedly in size it consisted of small follicles and individual follicular cells with PAS-positive colloid in a microfollicle in the cytoplasm (fig. 11). The mild increase of connective tissue in the interstitium appeared to be primarily the result of condensation of the normal stroma. Occasionally thyroid glands with idiopathic follicular atrophy had either an encapsulated microadenoma (3 dogs) or a non-encapsulated area of nodular hyperplasia of follicular cells (1 dog). Accumulations of few inflammatory cells were present only in association with a microadenoma.
The early lesion of idiopathic follicular atrophy by electron microscopy was interpreted to be degeneration of individual follicular cells lining thyroid follicles. Thyroid follicular cells undergoing degeneration had extensively dilated profiles of rough endoplasmic reticulum, swollen mitochondria with disrupted cristae and myelin bodies, an electron-dense cytoplasm, and irregularly shrunken nucleus (fig. 12). There was invagination of the follicular basement membrane and collagen fibers around the degenerate follicular cells. The remaining follicular cells were hypertrophic with a large cytoplasmic area, numerous profiles of rough endoplasmic reticulum, a well developed Golgi apparatus, long microvilli or pseudopodia, and occasional cytoplasmic projections on the apical surface extending into the colloid. The cytoplasm of some vacuolated follicular cells was filled with profiles of rough endoplasmic reticulum that were markedly distended by a finely granular material. Adjacent distended profiles of rough endoplasmic reticulum often had fused following disruption of their membranes.

The advanced stage of follicular atrophy was characterized ultrastructurally by a lack of normal thyroid follicles and the presence of microfollicles in the cytoplasm of individual follicular cells (fig. 13). These remaining hypertrophic follicular cells formed small nests and were closely arranged along capillaries. The microfollicles appeared to form by an invagination of the apical surface of the follicular cells. Long microvilli extended into the colloid and membrane-limited colloid droplets were present in the cytoplasm near the microfollicle, suggesting endocytotic activity. Degenerate follicular cells with poorly defined plasma membranes, an irregularly shrunken nucleus, markedly dilated profiles of rough endoplasmic reticulum, and swollen mitochondria with
disrupted cristae were scattered in the interstitium. Macrophages with membrane-limited vacuoles containing myelin bodies, other membranous debris, and lysosomes were present near the degenerate follicular cells. The basement membrane was focally thickened around remaining follicular cells and electron-dense deposits were present in the basement membrane of follicular cells at the periphery of a microadenoma in 1 dog. Thyroid C-cells were normal ultrastructurally and had many membrane-limited secretory granules in the cytoplasm.

**DISCUSSION**

This study revealed two distinct lesions in the thyroid gland of pet dogs with clinical hypothyroidism. Lymphocytic thyroiditis observed in pet dogs was more extensive than the multifocal lympho-plasmocytic infiltration of the thyroid gland reported in laboratory beagles that was not associated with significant disturbance of function (4,19). Lymphocytic thyroiditis appeared to progress to an end-stage form in pet dogs with replacement of thyroid parenchyma by a proliferation of fibrous connective tissue. In contrast, the early stage of idiopathic follicular atrophy was characterized by loss of thyroid follicles and gradual replacement by adipose connective tissue. In the advanced stage the thyroid gland consisted principally of adipose connective tissue with only a few small follicles and individual follicular cells, and markedly reduced circulating levels of thyroxine.

The histopathologic lesions in lymphocytic thyroiditis of laboratory beagles consisted of a multifocal infiltration of lymphocytes, plasma cells, and scattered macrophages in the interstitium with the formation of occasional
germinal centers but with an absence of fibrotic changes. Lymphocytic thyroiditis in laboratory beagles does not appear to be progressive but appears to reach a state at which the lesion becomes static for many years. No clinical signs suggestive of thyroid disease were observed in beagles (5).

The thyroid lesion most often detected in clinically hypothyroid dogs with lymphocytic thyroiditis appeared to resemble the autoimmune thyroiditis in human beings (1,15). A previous study revealed that 48% of pet dogs with hypothyroidism had circulating thyroglobulin autoantibodies but infrequent (4%) microsomal antibodies. Baseline circulating levels of triiodothyronine and thyroxine were either low or in the normal range but with an impaired ability to respond to a challenge by exogenous TSH (7). Signs of hypothyroidism and subnormal thyroid function have been reported in the later stage of lymphocytic thyroiditis in human beings (6,10).

Chickens of the Obese strain (OS) of White Leghorns have a naturally occurring, hereditary form of autoimmune lymphocytic thyroiditis. Hormonal bursectomy of OS embryos prevents the development of thyroiditis and neonatal thymectomy leads to a significant increase in the severity of thyroiditis (22). Thymectomy in newborn Buffalo rats has been reported to increase the incidence of thyroiditis and to reduce the age of onset (18). It was concluded from these studies that thymus-derived cells failed to suppress the autoimmune reaction and resulted in the development of thyroiditis. Current evidence suggests that the cell destruction observed in lymphocytic thyroiditis is related to thyroid autoantibodies reacting with the plasma membrane of follicular cells thereby allowing K cells to kill the target cell (16).
Many of the follicular lesions in pet dogs with lymphocytic thyroiditis appeared to be the result of lymphocytes and plasma cells migrating between thyroid epithelial cells. This migration resulted in the separation of adjacent follicular cells from the basement membrane, desquamation of follicular cells and migration of lymphoid cells into the lumen, and eventually destruction of the follicle. Although many of the thyroid lesions in dogs with thyroiditis appeared to result from lymphoid infiltration into follicles in later stage of the disease, degenerative changes of follicular cells have been reported in the OS chicken before any demonstrable lymphoid infiltration of the thyroid gland (21). To compensate for the progressive destruction of thyroid follicles by the inflammatory cells and the decreased production of thyroid hormones follicular cells in the remaining follicles underwent hypertrophy, presumably in response to an increased secretion of TSH.

Oxyphil cells (oncocyes) with an abundant eosinophilic cytoplasm were observed in thyroids of dogs with long-standing lymphocytic thyroiditis. Histochemical and ultrastructural evaluation of oxyphilic cells by other investigators has demonstrated abundant oxidative enzymes but a sparcity of rough endoplasmic reticulum, suggesting an imbalance between the respiratory and synthetic activities of the cell (15). Thyroid hormone synthesis appeared to be severely compromised in these modified follicular cells. It has been suggested that the occurrence of oxyphilic cells in thyroid glands may be related to aging or overstimulation (15).

The remaining follicular epithelial cells in dogs with lymphocytic thyroiditis often were surrounded by increased amounts of an altered basement membrane that was predisposed to the precipitation of electron-dense deposits.
These electron-dense deposits were similar to those reported by Kalderon and Bogaars (8) to represent immune complexes in human beings with Hashimoto's thyroiditis. Thyroid antigens from disrupted follicles bind to antibodies produced by stimulated plasma cells in the thyroid gland (11,13). However, since these electron-deposits first appeared when the thyroid lesions were advanced in the OS chicken, their role in the pathogenesis of autoimmune thyroiditis remains unclear (9).

Follicular atrophy of unknown etiology was a frequent thyroid lesion in dogs with clinical hypothyroidism. The lesion appeared to develop from a degeneration of individual follicular cells, subsequent desquamation into the colloid or interfollicular area, progressive reduction in follicular size, and replacement by adipose connective tissue. Idiopathic follicular atrophy was a primary degenerative disease of the thyroid and distinctly different from the trophic atrophy of follicular cells observed secondary to diminished secretion of TSH. Under these conditions thyroid follicles were lined by a low cuboidal epithelium and distended by uniformly dense PAS-positive colloid with little evidence of endocytosis in response to diminished TSH secretion (2). Ultrastructurally, the progressive degeneration of follicular cells in idiopathic follicular atrophy and formation of microfollicles in individual cells were markedly different lesions than reported in trophic atrophy of follicular cells as a result of diminished TSH stimulation (2). Thyroid follicles were enlarged but lining follicular cells had a diminished cytoplasmic area containing a few short profiles of rough endoplasmic reticulum, a small Golgi apparatus, and sparse short microvilli on the apical surface.
In the advanced stage of idiopathic follicular atrophy, the atrophic thyroid gland was composed of either of small follicles or individual follicular cells containing microfollicles in their cytoplasm. Microfollicles have been reported in normal fetal and neonatal animals, and coalesce to form thyroid follicles (3). In idiopathic follicular atrophy, the microfollicles appeared to develop from an invagination of the apical surface of the follicular cells. Microfollicles were interpreted to represent an attempt by the remaining viable follicular cells to continue the biosynthesis of thyroid hormones in the absence of normal follicles. Secretion of thyroglobulin into a follicular lumen is necessary for the iodination of attached tyrosyl residues and coupling of iodothyronines to form triiodothyronine and thyroxine (20).

The results of this investigation indicated that primary diseases of the thyroid gland, especially lymphocytic thyroiditis and idiopathic follicular atrophy, were responsible for clinical hypothyroidism in pet dogs. Lymphocytic thyroiditis in pet dogs resembled naturally occurring lymphocytic thyroiditis in the OS strain of White Leghorn chickens and Hashimoto's thyroiditis in human beings. The morphology of the thyroid lesion and frequent occurrence of circulating thyroglobulin autoantibodies (7) suggested that lymphocytic thyroiditis in pet dogs was immune mediated. Follicular atrophy was a degenerative lesion of follicular cells of unknown etiology not associated with inflammatory destruction in the thyroid gland.

**SUMMARY**

Thyroid lesions were evaluated in pet dogs with hypothyroidism by light and electron microscopy. Lymphocytic thyroiditis (7 dogs) was characterized by
a diffuse infiltration of the thyroid gland by lymphocytes, plasma cells and macrophages with the formation of occasional lymphoid nodules and destruction of follicles. Thyroiditis appeared to progress to an end-stage form where the majority of the thyroid was replaced by fibrous connective tissue. The basement membrane around follicles was thickened and had electron-dense deposits. The morphology of the thyroid lesions and frequent occurrence of circulating thyroglobulin autoantibodies suggested lymphocytic thyroiditis in pet dogs was immune mediated.

Idiopathic follicular atrophy (9 dogs) was characterized by a marked loss of thyroid parenchyma and replacement by adipose connective tissue. Degeneration of individual follicular cells was present in the early stage with desquamation into the colloid and interfollicular area. The majority of the thyroid gland consisted of adipose connective tissue with either interspersed small follicles or individual follicular cells that had an extensively dilated rough endoplasmic reticulum, a large Golgi apparatus, and intracytoplasmic microfollicles in the advanced stage. Follicular atrophy was a degenerative lesion of follicular cells of unknown etiology not associated with inflammatory destruction in the thyroid gland.
Figure 2.1: Severe lymphocytic thyroiditis with diffuse infiltration of the interstitium and follicular lumen (L) by lymphocytes, plasma cells, and macrophages. The remaining follicles are lined by columnar follicular cells and have a narrow lumen (arrow) with little colloid. Periodic acid–Schiff.
Figure 2.2: End-stage lymphocytic thyroiditis with extensive proliferation of connective tissue (C), a few remaining thyroid follicles (F), and microfollicles (arrows) within individual follicular cells. Focal accumulations of lymphocytes are present in the interstitium. Periodic acid-Schiff.
Figure 2.3: Lymphocyte (L) migrating between follicular cells (FC) toward the lumen (top) in severe lymphocytic thyroiditis. Microvilli (arrows) on the luminal surface of follicular cells are compressed by numerous lymphocytes in the follicular lumen. Basement membrane (B) of thyroid follicle is at the bottom.
Figure 2A: Markedly thickened, electron-dense basement membrane (arrows) around a degenerating follicular cell with a microfollicle (MF) in severe lymphocytic thyroiditis. Long microvilli (arrowheads) project into the lumen.
Figure 2.5: Electron-dense deposits (arrows) between follicular cells and the basement membrane (B).
Figure 2.6: Oxyphilic cells with numerous large mitochondria (M) and short profiles of rough endoplasmic reticulum (E) in the thyroid gland of a dog with chronic lymphocytic thyroiditis.
Figure 2.7: End-stage lymphocytic thyroiditis with numerous collagen fibers (CF) surrounding a small group of follicular cells. The hypertrophied follicular cells have dilated profiles of rough endoplasmic reticulum (E), a prominent Golgi apparatus (G), large lysosomal bodies, and an intracytoplasmic microfollicle (MF).
**Figure 2.8:** Early stage of follicular atrophy with a loss of thyroid follicles and replacement by adipose connective tissue (A) in one part of the thyroid but adjacent follicles (F) are unaffected. HE.
Figure 2.9: Loss of thyroid follicles and replacement by adipose connective tissue (A) in early follicular atrophy. The remaining thyroid follicles are small and lined by columnar follicular cells but contain little colloid (C). Degenerate follicular cells are present in the lumen and between follicles (arrows). HE.
Figure 2.10: Severe follicular atrophy in a dog with hypothyroidism. Both thyroid lobes were composed predominately of adipose connective tissue (A) with only occasional groups of small follicles containing little colloid (C). HE.
Figure 2.11: Severe follicular atrophy in a dog with hypothyroidism illustrating small follicles (F) and individual follicular cells with colloid-containing microfollicles (arrows). Periodic acid-Schiff.
Figure 2.12: Early stage of follicular atrophy illustrating degenerate changes in an individual follicular cell lining a thyroid follicle. The nucleus (N) of the degenerate follicular cell is irregularly shrunken and the cytoplasm is more electron-dense containing vacuolated mitochondria (arrows), extensively dilated rough endoplasmic reticulum (E), and numerous dense bodies. A bundle of collagen fibers (CF) is present near the degenerate follicular cell.
**Figure 2.13:** Severe follicular atrophy illustrating a lack of normal follicle formation in the thyroid and the presence of microfollicles (MF) in the cytoplasm of individual follicular cells. The remaining follicular cells are hypertrophied and have extensively dilated profiles of rough endoplasmic reticulum (E) and well developed Golgi apparatuses (G).
CHAPTER III

EXPERIMENTAL THYROIDITIS AND LOCAL

GRAFT-VERSUS-HOST REACTION IN DOGS

INTRODUCTION

Naturally occurring lymphocytic thyroiditis is an hereditary autoimmune disorder observed in laboratory beagles (8), the Obese strain of White Leghorn chickens (39), Buffalo rats (33), and in human beings (7). The pathogenesis of this autoimmune lesion has not been completely defined. The most widely accepted hypothesis is related to a malfunction of the immunoregulatory system (9) or other mechanisms which bypass the need for specific helper T lymphocytes (1). Autoimmune lymphocytic thyroiditis has been associated with other autoimmune diseases (6), an increased iodine intake (17,26), and following congenital viral infections in human beings (40). Experimental lymphocytic thyroiditis has been induced by the injection of thyroglobulin coupled with arsanilic and sulfanilic acids (38), by immunization of thyroid antigens admixed with complete Freund adjuvant (35), following immunization against streptococcus (36), and after treatment with immunosuppressive drugs (19,33).

Autoimmune hemolytic anemia, membranous glomerulonephritis, anti-nuclear and other autoantibodies have been reported in association with
experimental systemic graft-versus-host reaction (GVHR) (5). Bone-marrow transplantation performed in man for treatment of leukemia, immunodeficiency or aplastic anemia often is complicated by a systemic GVHR followed by autoimmune diseases such as lupus erythematosus, Sjogren's syndrome, and scleroderma (13,31). Autoimmune thyroiditis or circulating thyroid autoantibodies have not been reported in association with systemic GVHR (14).

Previous investigations from our laboratory demonstrated that lymphocytic thyroiditis in hypothyroid pet dogs was characterized by a multifocal interstitial infiltration of lymphocytes, plasma cells and macrophages with destruction of thyroid follicles and the presence of electron-dense deposits in the follicular basement membrane, resembling antigen-antibody complexes (10). Forty-eight percent of pet dogs with clinical hypothyroidism had circulating thyroglobulin autoantibodies as determined by chromic chloride hemagglutination (11). Specific damage to follicular cells by iodine irradiation lead to massive destruction of thyroid follicles but it did not develop a progressive thyroiditis (7). Lymphocytic thyroiditis has been produced by chemicals, drugs and thyroglobulin-adjuvant immunization. All of these methods are artificial in nature and have not completely elucidated the pathogenesis of naturally occurring lymphocytic thyroiditis. In this investigation, we report the induction of autoimmune thyroiditis by an immunological mechanism that does not involve immunization with thyroid antigens in adjuvant. The specific objectives of this study were: 1) to induce a local GVHR in one thyroid lobe by the intrathyroidal injection of 50 million lymphocytes and to evaluate thyroid lesions by light and electron microscopy at one week and one month after the injection, 2) to compare thyroid lesions of GVHR with thyroid lesions observed in the non-
injected contralateral thyroid lobe, 3) to determine the effect of the hemithyroidectomy of the lobe undergoing GVHR on the development of thyroid lesions in the non-injected contralateral thyroid lobe.

MATERIALS AND METHODS

ANIMALS

Fifteen male, adult (2 to 4 years of age), mixed-breed dogs were used in this study. The dogs were subdivided in 3 groups of 5 dogs each. Blood samples were collected before treatment and once a week for 4 weeks after treatment for assay of circulating levels of triiodothyronine (T₃) and thyroxine (T₄), thyroglobulin autoantibodies, and lymphocyte-blast transformation. All dogs received 0.1 mg./lb. of acepromazine (Ayerst Laboratories, New York, New York) intramuscularly before the intrathyroidal injection. They were anesthetized by the intravenous administration of 10 mg./lb. of pentobarbital sodium (Nembutal Sodium, Abbott Laboratories, Chicago, Illinois). A 5 cm. long incision was performed on the middle line in the ventral cervical region.

The left thyroid lobe was isolated in dogs from groups 1 and 2 and injected with 50 X 10⁶ of non-sensitized peripheral blood lymphocytes in 0.5 ml. of physiologic saline from a clinically normal unrelated dog (Table 1). The injection site was identified by a suture (Prolene 3-0, Ethicon, Somerville, New Jersey) in the thyroid capsule.

In group 1, the left and right thyroid lobes were collected 4 weeks post-injection (Table 1). The left thyroid lobe from dogs in group 2 was removed one week after intrathyroidal injection and the right thyroid lobe 3 weeks after the hemithyroidectomy. In order to determine the effects of the injection on the
thyroid tissue, a third group of 5 dogs were injected with a similar volume (0.5 ml.) of normal canine serum by a 27 gauge needle. This control group was shared with another experiment and reported elsewhere (12). Thyroids from dogs injected with normal serum did not have significant lesions 1 week and 1 month after intrathyroidal injection. Thyroid lobes from all dogs were cut longitudinally through the injection site. One-half of each thyroid lobe was evaluated by light microscopy and tissue was collected from the other half for evaluation by electron microscopy.

SOURCE OF LYMPHOCYTES FOR INJECTION

Peripheral blood lymphocytes were separated from fresh heparinized whole blood from a clinically normal dog by centrifugation (1800 r.p.m. for 40 minutes) over a commercial lymphocyte separation medium (LSM, Litton Bionetics, Kensington, Maryland). The purified mononuclear cell layer was harvested, washed 2 times with phosphate-buffered saline, washed once with normal saline, and resuspended in sterile saline at a concentration of $50 \times 10^6$ cells per 0.5 ml.

HORMONE ASSAYS

Serum triiodothyronine (T$_3$) and thyroxine (T$_4$) were determined by radioimmunoassay (V.K. GanJam, Unpublished data).

CHROMIC CHLORIDE PASSIVE HEMAGGLUTINATION (CCH) TEST

Thyroglobulin autoantibodies were determined by the CCH test. The CCH test was a modification of the procedure reported by Poston (29). Sheep red blood cells were washed 3 times with isotonic saline. The purification of canine thyroglobulin has been described previously (11). To coat the sheep red blood
cells with canine thyroglobulin, 0.5 ml. of 0.27 M piperazine buffer was added to a 40 ml. centrifuge tube. The 0.1 ml. of canine thyroglobulin (1.0 mg./ml.), 0.1 ml. of packed washed erythrocytes, and 0.2 ml. of $5 \times 10^{-3}$ M chromic chloride ($\text{CrCl}_3$) were added sequentially to the tube. The suspension was agitated gently for 4 to 5 minutes. The reaction was stopped by the addition of 30 ml. of saline and the cells were washed 3 times in saline by centrifuging at 1250-1350 r.p.m. for 8 minutes. Sheep red blood cells were used at a cell concentration of 0.66%. The test was performed with microtiter "V" bottom plates.

LYMPHOCYTE-BLAST TRANSFORMATION ASSAY (LBT)

Heparinized blood (10 ml.) containing 20 units of preservative-free heparin per ml. was collected before the intrathyroidal injection and every week thereafter for 4 weeks. The blood samples were always taken at the same time of the day (between 9 and 10 A.M.).

Blood was diluted with 2 volumes of calcium and magnesium ion-free, phosphate-buffered saline (TC-PBS) at pH 7.5. Lymphocytes were isolated as above and the mononuclear leukocytes at the plasma-LSM interface were carefully aspirated and suspended in an equal volume of TC-PBS. Cells were washed 3 times in TC-PBS (1000 r.p.m. for 10 minutes) and were suspended in Eagle's minimum essential medium with Spinner salts (EMEM-S) containing 1% antibiotics (penicillin, 2000 units/ml.; streptomycin, 10 mg./ml.; and nystatin, 5000 units/ml.). The final preparation was adjusted to $10^6$ mononuclear cells/ml with media (EMEM-S) containing 20% heat-inactivated homologous normal canine serum. The same pool of canine serum was used throughout the experiment.
The lymphocyte blast transformation (LBT) assay was performed according to the method described for the dog by Krakowka et al. (21). The peripheral blood mononuclear cells were cultured in triplicate in flat bottom microtiter plates at $10^5$ viable cells per well with an equal volume of phytohemagglutinin-P diluted 3 µg./ml. in media or an equal volume of growth media. The cultures were incubated 52 hours at 30°C in a humidified 5% CO$_2$ incubator. Tritiated thymidine (0.5 µCi) was added to each culture well and incubated for 18 hours. Subsequently, cells were collected and washed with a multiple automatic sample harvester. Radioactivity incorporated into newly synthesized cellular nucleic acid was trapped on glass filter paper which was dropped into scintillation vials filled with a toluene-base cocktail (Permablend II, Packard Instruments Co., Downers Grove, Illinois). Counts per minute were determined for each culture in a liquid beta scintillation counter (Beckman Model LS 8100). The degree of stimulation of lymphocytes by phytohemagglutinin-P was measured in counts per minute (CPM). A stimulation index (SI) was calculated by dividing stimulated counts by unstimulated counts (Table 2).

The LTB assay also was performed using peripheral blood lymphocytes from 5 normal dogs incubated with mitogen and sera was collected from experimental dogs before (day 0) and after the intrathyroidal injection of immunocompetent cells.

STATISTICAL ANALYSIS

Data from LBT and thyroid hormone assays were analysed statistically by the one-way analysis of variance and by the less significant difference contrast (LSD). This is a Student's "t" test which takes in account the multiple comparison between groups (24).
LIGHT AND ELECTRON MICROSCOPY

A longitudinal section of each thyroid lobe through the injection site was fixed in Bouin's fluid, and multiple sections were cut at 4 μ, and stained with hematoxylin and eosin. Ten to 20 one mm. cubes from an area in close proximity to the injection site and 2 other areas between the injection site and pole of each thyroid lobe were collected for electron microscopy, fixed in ice cold 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, and post-fixed with 1.33% osmium tetroxide in s-collidine at pH 7.2. Tissue blocks were dehydrated in ascending concentrations of ethanol, transferred to propylene oxide, and embedded in Epon 812. One micron thick sections were cut from each block and stained with toluidine blue for light microscopic evaluation and selection of the most appropriate area for sectioning. Thin sections were cut at 600 to 800 Å and stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope.

RESULTS

THYROID HORMONE LEVELS

Circulating levels of triiodothyronine (T₃) did not change significantly between baseline and days 7, 14, 21 and 28 after intrathyroidal injection of 50 x 10⁶ lymphocytes in the 2 groups of dogs (Table 3). The blood level of thyroxine (T₄) increased significantly (P < 0.05) on day 14 and 28 compared to day 0 in dogs receiving 50 x 10⁶ lymphocytes (group 1). Dogs injected with 50 x 10⁶ lymphocytes and subjected to hemithyroidectomy on day 7 (group 2) did not have significant changes in serum thyroxine. Circulating thyroid hormone levels (T₃ and T₄) in group 1 compared to group 2 were not significantly different.
IMMUNOLOGIC STUDIES

The CCH test revealed a consistent lack of thyroglobulin autoantibodies in serum from the 2 groups of experimental dogs. A positive control serum (titer of 1:1280) obtained from a rabbit immunized with canine thyroid extract in complete Freund adjuvant was consistently positive indicating that the CCH assay was capable of detecting antibody activity in the experimental sera.

Lymphocyte blast transformation results (CPM and SI) on day 7 following injection were significantly decreased (P<0.05) from the baseline in group 1 (Table 2). The difference between group 1 and 2 for CPM and SI was not statistically significant. The percent increase above the baseline response was used to illustrate the effect of an intrathyroidal injection of lymphocytes on the incorporation of tritiated thymidine by peripheral blood mononuclear cells (Fig. 1). The inhibitory effect of GVHR on LBT after induction was greatest on day 7 for group 1 and on day 14 for dogs in group 2. This immunosuppression was transient for dogs in both groups. Lymphocyte blast transformation returned to 80% of the baseline response on day 14 and was approximately 100% of baseline on day 28. The LBT suppression observed in group 2 was immediately followed by a sharp increase in mitotic activity. The peak increase (2 times baseline) on day 28 was not significantly different from the baseline response or from group 1. Normal peripheral blood lymphocytes incubated with serum collected either on day 7 (group 1) or day 14 (group 2) did not have significantly depressed mitotic activity compared to normal lymphocytes incubated with pre-injection serum on day 0.

HISTOPATHOLOGIC EVALUATION OF THE THYROID

Injected Thyroid Lobe - One Week. Thyroids injected with lymphocytes (group 2) were infiltrated by multifocal accumulations of lymphocytes,
macrophages and few plasma cells (5 dogs) (Fig. 2). Inflammatory cells were often closely associated with small vessels. Many polymorphonuclear cells were present in vessels which often filled the lumen. A few thyroid follicles were surrounded by an intense infiltration of mononuclear cells (Fig. 3). Thyroid follicles were lined by cuboidal to columnar epithelial cells with a homogeneous or vacuolated colloid. Isolated degenerate follicular cells with a densely eosinophilic cytoplasm and pyknotic nuclei were present in the follicular wall and colloid.

**Injected Thyroid Lobe - One Month.** Thyroid lesions observed one month after the intrathyroidal injection of lymphocytes (group 1) consisted of extensive multifocal areas of necrosis in all 5 dogs. Many thyroid follicles were completely disrupted with escape of colloid into the interstitium (Fig. 4). Other thyroid follicles were lined by isolated degenerate follicular cells with a densely eosinophilic cytoplasm and pyknotic nuclei. Degenerate follicular cells also were present in the colloid and in the interstitium. A few foci of lymphocytes and plasma cells were scattered throughout the thyroid lobe.

**Non-Injected Thyroid Lobe - One Month.** Thyroid glands one month after the intrathyroidal injection of lymphocytes in the opposite gland (group 1) had multifocal accumulations of lymphocytes and many plasma cells (3 of 5 dogs). The infiltration was especially prominent around small vessels (Fig. 5). Many thyroid follicles were lined by degenerate follicular cells with a densely eosinophilic cytoplasm and pyknotic nuclei (4 dogs) (Fig. 6). Degenerate follicular cells also were present in the colloid.
The non-injected thyroid lobe (group 2), following hemithyroidectomy at one week of the injected lobe undergoing GVHR, had a focal infiltration of polymorphonuclear cells (eosinophils and neutrophils), lymphocytes, plasma cells, mast cells, and macrophages around large vessels (2 dogs) (Fig. 7). The inflammatory lesions were associated with destruction of adjacent thyroid follicles. The remaining 3 dogs had only isolated degenerate follicular cells.

ULTRASTRUCTURAL EVALUATION OF THE THYROID

**Injected Thyroid Lobe - One Week.** Thyroids were characterized by a perivascular infiltration of small and large lymphocytes, macrophages and plasma cells. The lymphocytes had many free ribosomes, occasional mitochondria in the cytoplasm, and clumped nuclear chromatin (Fig. 8). Vascular lumens often contained many polymorphonuclear cells but endothelial cells appeared to be normal. Bundles of collagen fibers were present in the perivascular and interfollicular spaces. Follicular cells were cuboidal to tall columnar with many microvilli on the apical surface, short profiles of rough endoplasmic reticulum, many lysosomal bodies, and normal mitochondria (Fig. 9). The basement membrane was focally reduplicated and contained cellular debris.

**Injected Thyroid Lobe - One Month.** Isolated degenerate follicular cells were present in the follicular wall that had dilated profiles of rough endoplasmic reticulum and swollen mitochondria with disrupted cristae (Fig. 10). The nucleus of degenerate follicular cells had irregularly clumped chromatin and electron-lucent areas were present in the cytoplasm with few organelles and containing a finely granular material (Fig. 11). Occasional thyroid follicles were lined completely by degenerate follicular cells. Necrotic
cellular debris was admixed with colloid in many thyroid follicles (Fig. 12). Lymphocytes had occasionally infiltrated between thyroid follicular cells but were not present in the follicular lumen. The basement membrane was focally reduplicated. Discrete electron-dense deposits were present between the thyroid follicular cells and basement membrane in the most severe thyroid lesions (Fig 10). Bundles of collagen fibers were present in the interfollicular area.

**Non-Injected Thyroid Lobe - One Month.** Thyroid follicles were lined by cuboidal to columnar follicular cells with slightly dilated profiles of rough endoplasmic reticulum, a few lysosomal bodies, and long microvilli. The basement membrane was focally reduplicated. Thickening of the basement membrane with electron-dense deposits was associated with the most severe inflammatory lesion by light microscopy (Fig. 13). Isolated degenerate thyroid follicular cells with dilated profiles of rough endoplasmic reticulum, swollen mitochondria with disrupted cristae, and clumped nuclear chromatin were present lining scattered follicles (Fig. 14). Accumulations of plasma cells often were present near small vessels (Fig. 15).

The non-injected thyroid lobe, following hemithyroidectomy of the lobe undergoing GVHR, had a focal interstitial infiltration of lymphocytes and occasional mast cells at 4 weeks (group 2). The follicular cells were cuboidal to low columnar with a focal reduplication of the basement membrane containing cellular debris. Isolated follicular cells had irregularly clumped nuclear chromatin and dilated profiles of rough endoplasmic reticulum. Bundles of collagen fibers were present in the interstitium.
DISCUSSION

The results of this study demonstrated that the intrathyroidal injection of immunocompetent cells resulted in thyroid lesions similar to a local GVHR reported in other organs (18,34). Thyroid lesions one week post-injection were characterized by multifocal infiltration of lymphocytes, macrophages and plasma cells, and the presence of isolated or small groups of degenerate follicular cells in the follicular wall and colloid. Extensive necrosis of follicular cells, scattered foci of inflammatory cells, and electron-dense deposits between follicular cells and the basement membrane were observed 4 weeks post-injection. In addition, the non-injected contralateral thyroid gland at 4 weeks was infiltrated by lymphocytes, macrophages, and many plasma cells, and associated with disseminated foci of degenerate follicular cells. Focal electron-dense thickenings of the basement membrane were observed with the most severe inflammatory lesions. The non-injected thyroid lobe following hemithyroidectomy at one week of the thyroid gland undergoing GVHR had isolated foci of polymorphonuclear cells, lymphocytes, and macrophages or no significant thyroid lesion. The inhibitory effect of GVHR on LBT-assay was observed on day 7 or 14. Circulating thyroid autoantibodies were not detected by the CCH test. Circulating levels of $T_3$ and $T_4$ remained in the normal range except that $T_4$ was increased on days 14 and 28 in dogs receiving only the intrathyroidal injection of lymphocytes.

Inflammatory lesions present in the non-injected thyroid lobe shared certain features with naturally occurring lymphocytic thyroiditis in laboratory beagles (37) and experimental autoimmune thyroiditis in dogs induced by immunization with thyroid extract and complete Freund's adjuvant (35). The
dogs developed multifocal lymphocytic thyroiditis with low circulating titers of thyroid antibodies after 16 to 26 months. Antibodies against thyroglobulin, microsomal antigen and a second colloid antigen have been demonstrated in laboratory beagles with lymphocytic thyroiditis (24). An effector cellular immune response (cytotoxic T cells) does not appear to play a direct role in the pathogenesis of autoimmune thyroiditis (4,28).

In systemic GVHR, the sequence of events following specific stimulation of donor lymphocytes by foreign antigens are proliferation and differentiation to cytotoxic T cells and memory cells of recipient histocompatibility antigen specificity. The developing responses cause immune-mediated damage to the host (16). Circulating autoantibodies also occur in systemic GVHR (5).

Thyroidectomy of the lobe undergoing GVHR resulted either in no significant lesions in the non-injected contralateral thyroid lobe or small focal infiltrations of polymorphonuclear cells, lymphocytes, and macrophages. These results suggested that a local intrathyroidal GVHR must be sustained for several weeks in order to produce thyroid lesions resembling autoimmune thyroiditis in the contralateral non-injected lobe. The hypotheses most widely accepted for the pathogenesis of autoimmune disorders suggest they are related to a malfunction of the immunoregulatory system (suppressor T cells) (9) or other mechanisms which bypass the need for helper T lymphocytes binding to autoantigens (1). Normally, autoantibody formation does not occur because T lymphocytes are tolerant to low dose of autoantigens. During a systemic GVHR, non-specific factors released by specifically activated T lymphocytes stimulate B lymphocytes to form antibodies (2,23).

As a consequence of antigenic stimulation, many B lymphocytes of host origin accumulate in a local GVHR (30). B lymphocytes that bind thyroid
antigens are normally found in circulation (3). During an intrathyroidal GVHR, host B lymphocytes capable of reacting with thyroid antigens may reach the thyroid gland. Subsequent proliferation of these cells within the thyroidal environment, presumably under helper T cell influence, could result in the local production of antithyroid antibodies. IgG-containing cells of host origin have been demonstrated in a local renal GVHR (34). In our experimental dogs electron-dense deposits, resembling antigen-antibody complexes, were present in the thyroid lobe undergoing GVHR one month following injection and in the non-injected contralateral lobe. Locally produced autoantibodies appeared to be formed in the thyroid and rapidly absorbed by the contralateral thyroid since circulating thyroglobulin autoantibodies were not be detected by the CCH test. Alternatively, thyroglobulin-specific host B lymphocytes may re-enter the circulation and colonize the non-injected contralateral gland. In the presence of thyroid antigens, these cells would produce autoantibodies locally thereby inducing the lesions observed in the contralateral thyroid lobe in the experimental dogs. Migration of $^{51}$Cr-labeled donor lymph node cells have been reported in the thyroid gland of rats undergoing a local GVHR (20). Donor lymphocytes spread from the injected thyroid to involve the regional lymph nodes and spleen but did not infiltrate the non-injected contralateral thyroid lobe. The lesions found in the non-injected lobe were degenerative with an accumulation of cells in the colloid.

In thyroidal GVHR two distinct immunologic phenomena were demonstrated in experimental dogs. These were: 1) the proliferation of immunocompetent cells resulting in the development of inflammatory lesions similar to autoimmune lymphocytic thyroiditis in the non-injected contralateral thyroid...
lobe, and 2) immunosuppression represented by a decreased proliferative response to mitogen as measured in the LBT assay. Suppression of both cell-mediated and humoral immune response has been demonstrated in animals with GVHR (22, 27). Suppressor T lymphocytes originating from the proliferation of donor lymphocytes appear to be the mechanism of GVHR-associated immunosuppression (31).

In conclusion, the results of this investigation demonstrated that a local GVHR in one thyroid lobe induces lesions similar to naturally occurring autoimmune thyroiditis in the non-injected contralateral thyroid lobe in dogs. The failure to develop lymphocytic thyroiditis following early (1 week) thyroidectomy of the lobe undergoing GVHR indicates that a sustained intrathyroidal GVHR was necessary to induce the autoimmune lesions in the non-injected contralateral thyroid lobe.

**SUMMARY**

Fifty million allogenic canine lymphocytes were injected in the left thyroid gland of 10 dogs. Thyroidectomy of the left thyroid was performed at 1 week and 4 weeks after injection. The intrathyroidal injection of immunocompetent cells resulted in local graft-versus-host reaction (GVHR) within the thyroid gland. Multifocal infiltration of lymphocytes, plasma cells and macrophages associated with scattered foci of isolated or small groups of degenerate follicular cells were present in the follicular wall and colloid on day 7. Thyroid lesions at one month after injection were characterized by extensive destruction of follicular cells, disseminated foci of mononuclear cell infiltration, and electron-dense deposits between follicular cells and the
basement membrane. The contralateral, non-injected thyroid lobe 4 weeks following injection was infiltrated by lymphocytes, macrophages, and many plasma cells. The inflammatory lesions were associated with multifocal areas of degeneration of follicular cells and focal electron-dense thickenings of the basement membrane. The non-injected thyroid lobe following hemithyroidectomy one week after injection of the thyroid gland undergoing GVHR, either had a focal infiltration of polymorphonuclear leukocytes, lymphocytes, macrophages, and mast cells or no significant lesions. There were no detectable circulating thyroid autoantibodies by the chronic chloride hemagglutination test. An inhibitory effect of thyroidal GVHR on the LBT-assay was observed on day 7 or day 14. Circulating levels of T₃ and T₄ remained in the normal range except that T₄ increased on days 14 and 28 in dogs receiving only the intrathyroidal injection of lymphocytes. These results indicated that a local GVHR in one thyroid lobe induced lesions similar to naturally occurring autoimmune thyroiditis in the non-injected contralateral thyroid gland. The failure to develop lymphocytic thyroiditis following early (1 week) thyroidectomy of the lobe undergoing GVHR indicates that a sustained GVHR in the thyroid was necessary to induce the autoimmune lesion in the non-injected thyroid lobe.
Table 3.1 Experimental Design for the Induction of Thyroid GVHR and Experimental Lymphocytic Thyroiditis in Dogs.

<table>
<thead>
<tr>
<th>GROUP 1 (n=5 dogs)</th>
<th></th>
<th>GROUP 2 (n=5 dogs)</th>
<th></th>
<th>GROUP 3 (n=5 dogs)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Left Thyroid Lobe</strong></td>
<td><strong>Right Thyroid Lobe</strong></td>
<td><strong>Left Thyroid Lobe</strong></td>
<td><strong>Right Thyroid Lobe</strong></td>
<td><strong>Left Thyroid Lobe</strong></td>
<td><strong>Right Thyroid Lobe</strong></td>
</tr>
<tr>
<td>Injected 50 X 10^6 Lymphocytes</td>
<td>Not Injected</td>
<td>Inject 50 X 10^6 Lymphocytes</td>
<td>Not Injected</td>
<td>Injected with normal canine serum</td>
<td>Injected with normal canine serum</td>
</tr>
<tr>
<td>Collected 4 Wk. PI</td>
<td>Collected 4 Wk. PI</td>
<td>Collected 1 Wk. PI</td>
<td>Collected 4 Wk. PI</td>
<td>Collected 1 Wk. PI</td>
<td>Collected 4 Wk. PI</td>
</tr>
</tbody>
</table>

PI = Weeks post-injection of 50 million lymphocytes into the left thyroid lobe.
Table 3.2. Lymphocyte-Blast Transformation in Response to Phytohemagglutinin-P Expressed as Mean Counts/Min (CPM) and Stimulation Index (SI)\(^{a}\) in Dogs Following an Intrathyroidal Injection of 50 X 10\(^6\) Lymphocytes.

<table>
<thead>
<tr>
<th>Group(^{b})</th>
<th>Parameter</th>
<th>Days Post-Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>CPM</td>
<td>4502</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+354</td>
</tr>
<tr>
<td></td>
<td>SI(^{d})</td>
<td>104.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+20.5</td>
</tr>
<tr>
<td>2</td>
<td>CPM</td>
<td>4767</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+1515</td>
</tr>
<tr>
<td></td>
<td>SI(^{d})</td>
<td>106.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+20.2</td>
</tr>
</tbody>
</table>

\(^{a}\) Mean ± standard error.

\(^{b}\) Group 1 = thyroid gland injected with 50 x 10\(^6\) lymphocytes
Group 2 = thyroid gland injected with 50 x 10\(^6\) lymphocytes plus hemithyroidectomy at one week.

\(^{c}\) Significantly different from baseline p < 0.05.

\(^{d}\) Stimulation index = stimulated CPM/unstimulated CPM.
Table 3.3. Serum Triiodothyronine (T<sub>3</sub>) and Thyroxine (T<sub>4</sub>) in Dogs Receiving an Intrathyroidal Injection of 50 x 10<sup>6</sup> Lymphocytes.<sup>a</sup>

<table>
<thead>
<tr>
<th>Group&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hormone</th>
<th>Days Post-Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>T&lt;sub&gt;3&lt;/sub&gt; (ng/dl)</td>
<td>66.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+5.2</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>75.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+10.4</td>
</tr>
<tr>
<td>1</td>
<td>T&lt;sub&gt;4&lt;/sub&gt; (µg/dl)</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+0.08</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+0.10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± standard error.

<sup>b</sup> Group 1 = thyroid gland injected with 50 x 10<sup>6</sup> lymphocytes
Group 2 = thyroid gland injected with 50 x 10<sup>6</sup> lymphocytes plus hemithyroidectomy at one week.

<sup>c</sup> Significantly different from baseline at P < 0.05.
Figure 3.1. Lymphocyte-blast transformation in response to phytohemagglutinin-P expressed as percent of the baseline response in dogs receiving 50 X 10^6 lymphocytes.
LYMPHOCYTE BLAST TRANSFORMATION
% INCREASE (CPM) ABOVE BASELINE RESPONSE

DAYS POST INJECTION

LEFT THYROID INJECTED WITH 5 x 10^6 LYMPHOCYTES ●●
LEFT THYROID INJECTED WITH 5 x 10^6 LYMPHOCYTES ▲▲
+ LEFT HEMITHYROIDECTOMY AT 1 WEEK

N = 5 DOGS / GROUP

Fig. 1
Figure 3.2. Thyroid GVHR one week following injection of 50 million lymphocytes. There is an interstitial infiltration of lymphocytes, macrophages and plasma cells near vessels (V). Thyroid follicles (F) are lined by cuboidal to columnar follicular cells with many vacuoles in the colloid (arrow). Hematoxylin and eosin; X 315.
Figure 3.3. Thyroid GVHR one week following injection of 50 million lymphocytes. Many lymphocytes, macrophages, and plasma cells surround a thyroid follicle (F). Hematoxylin and eosin; X 315.
Figure 3.4. Thyroid GVHR at one month illustrating destruction of entire thyroid follicles (F). Individual or groups of degenerate follicular cells are present in the follicular wall and colloid (arrows). Hematoxylin and eosin; X 315.
Figure 3.5. Infiltration of lymphocytes, plasma cells and macrophages around small vessels (V) and adjacent thyroid follicles (F) in non-injected thyroid gland one month following injection of 50 million lymphocytes. Hematoxylin and eosin; X 315.
Figure 3.6. Degenerate follicular cells in the follicular wall (F) and colloid (arrow) in non-injected thyroid gland one month following injection of 50 million lymphocytes. Hematoxylin and eosin; X 315.
Figure 3.7. Interstitial infiltration of mast cells (arrowhead), polymorphonuclear cells (arrow), lymphocytes, plasma cells and macrophages near a vessel (V) in non-injected thyroid gland following hemithyroidectomy at one month. Hematoxylin and eosin; X 315.
Figure 3.8. Thyroid GVHR one week following injection of 50 million lymphocytes. There is a perivascular infiltration of lymphocytes (L), plasma cells (P) and macrophages (M), and a polymorphonuclear (PM) cell in the vessel lined by normal endothelial cells (E). Increased collagen fibers (CF) are present in the interstitium. X 2800.
Figure 3.9. Thyroid GVHR at one week following injection of 50 million lymphocytes. The follicular basement membrane was reduplicated (arrows) and contained cellular debris. Thyroid follicular cells have many microvilli (V) and lysosomal bodies (L), short profiles of rough endoplasmic reticulum, and normal mitochondria. X 2800.
Figure 3.10. Isolated degenerate follicular cell with dilated profiles of rough endoplasmic reticulum (E), swollen mitochondria (M) with disrupted cristae, and short microvilli (V) in thyroid GVHR at 1 month. Focal electron-dense deposits are present (arrows) in the follicular basement membrane. X 3400.
Figure 3.11. Thyroid GVHR at one month. Thyroid follicle lined by columnar follicular cells with long microvilli (V), markedly dilated rough endoplasmic reticulum (E), and a nucleus with irregularly clumped chromatin. Finely granular material and many colloid droplets are present in the apical cytoplasm. Thyroid from dog 1 month following injection of 50 million lymphocytes. X 3400.
Figure 3.12. Degenerate nuclei (N) and other cellular debris admixed with colloid in a thyroid follicle lined by flattened follicular cells (F). Thyroid GVHR 1 month following injection of 50 million lymphocytes. X 2800.
Fig. 12
Figure 3.13. Thyroid follicular cell in non-injected thyroid lobe at one month with tubular profiles of rough endoplasmic reticulum (E), few lysosomal bodies (L), and long microvilli (V). There are focal electron-dense thickenings of the basement membrane (arrow). X 4900.
Figure 3.14. Isolated degenerate thyroid follicular cell in thyroid GVHR at one month with dilated rough endoplasmic reticulum (E), swollen mitochondria with disrupted cristae (M), and few short microvilli (V) in the luminal surface. The adjacent basement membrane is focally thickened (arrows). X 4000.
Figure 3.15. Perivascular accumulation of plasma cells (P) and occasional lymphocytes (L) in the non-injected thyroid gland at one month. X 2800.
CHAPTER IV

EXPERIMENTALLY INDUCED LYMPHOCYTIC THYROIDITIS IN DOGS:
EFFECT OF INTRATHYROIDAL INJECTION OF THYROID AUTOANTIBODIES

INTRODUCTION

Naturally occurring lymphocytic thyroiditis has been reported in laboratory beagles (1) and pet dogs with clinical hypothyroidism (2). Previous investigations from our laboratory reported that 48% of hypothyroid dogs had circulating thyroglobulin autoantibodies (3). Thyroid lesions in hypothyroid dogs with autoimmune thyroiditis were characterized by a multifocal to diffuse interstitial infiltration of lymphocytes, plasma cells, and macrophages associated with destruction of thyroid follicles and the presence of electron-dense deposits in the follicular basement membrane, resembling antigen-antibody complexes. The mononuclear cell infiltrate in the thyroid of animals with autoimmune thyroiditis has been interpreted as evidence that the damage to follicular cells was due to cytotoxic T lymphocytes (4). However, Totterman (5) reported that there were as many B as T lymphocytes in the inflammatory infiltrate of the thyroid in human beings with Hashimoto's thyroiditis.

The traditional method for distinguishing between antibody-mediated and cell-mediated reactions has been by transfer experiments. Lymphocytic
thyroiditis has been transferred by serum (6,7,8), lymphocytes (9,10,11) or a combination of both (12) from donors with experimentally induced autoimmune thyroiditis. Normal peripheral lymphocytes incubated with serum containing thyroid autoantibodies from experimental (13) or naturally occurring lymphocytic thyroiditis (14) will lyse either sheep erythrocytes coated with thyroglobulin or normal thyroid cells in culture (15). However, there is little direct evidence for a role of this mechanism of follicular cell damage in naturally occurring lymphocytic thyroiditis even though the production of thyroid autoantibodies has been demonstrated in the thyroid gland (16). Thyroid autoantibodies could react with follicular cells and fix the complement in the complement-dependent cytotoxicity or collaborate with killer cells in the antibody-dependent cell-mediated cytotoxicity, they could form immune complexes with the binding of thyroid antigens and complement or be secondary to cellular injury with no significant role in the pathogenesis of autoimmune thyroiditis. The specific objectives of this investigation were: 1) to determine if thyroid lesions similar to autoimmune thyroiditis could be induced by the intrathyroidal injection of thyroglobulin autoantibodies from a dog with naturally occurring lymphocytic thyroiditis, and 2) to evaluate thyroid lesions by light and electron microscopy and correlate these findings with circulating thyroglobulin autoantibody titers, thyroid hormone levels, and the mitogenic response of peripheral lymphocytes as determined by the lymphocyte-blast transformation assay.
**Experimental Animals**

Ten male, adult (2 to 4 years of age), mixed-breed dogs were used in this study. The dogs were subdivided in 2 groups of 5 dogs each. Blood samples were collected before treatment and once a week for 4 weeks after treatment for assay of circulating levels of triiodothyronine (T₃) and thyroxine (T₄), thyroglobulin autoantibodies, and lymphocyte-blast transformation (LBT).

All dogs received 0.1 mg/lb of acepromazine (Ayerst Laboratories, New York, NY) intramuscularly before the intrathyroidal injection. They were anesthetized by the intravenous administration of 10 mg/lb of pentobarbital sodium (Nembutal Sodium, Abbott Laboratories, Chicago, IL). A 5 cm incision was made on the mid line in the ventral cervical region. Both thyroid lobes were exposed and injected with 0.5 ml of serum using a 27 gauge needle. The injection sites were identified by a suture (Prolene 3-0, Ethicon, Somerville, NJ) in the thyroid capsule.

The experimental group of 5 dogs received an intrathyroidal injection in both thyroid lobes of 0.5 ml of serum containing thyroglobulin autoantibodies (titer: 1:1280) from a laboratory beagle with naturally occurring lymphocytic thyroiditis. The control group of 5 dogs was injected in the left and right thyroid lobes with 0.5 ml of serum from a clinically normal dog with a negative titer for circulating thyroglobulin autoantibodies as determined by the chromic chloride hemagglutination test. A hemithyroidectomy of the left thyroid lobe was performed on all dogs 1 week after the intrathyroidal injection.
Thyroid lobes from all dogs were cut longitudinally through the injection site. One-half of each thyroid lobe was evaluated by light microscopy and tissue was collected from the other half for evaluation by electron microscopy.

**Hormone Assays**

Serum triiodothyronine ($T_3$) and thyroxine ($T_4$) were determined by radioimmunoassay (V.K. GanJam, unpublished data).

**Chromic Chloride Passive Hemagglutination (CCH) Test**

Thyroglobulin autoantibodies were determined by the CCH test. The CCH test was a modification of the procedure reported by Poston (17). Sheep erythrocytes were washed 3 times with isotonic saline. The purification of canine thyroglobulin has been described in a previous experiment (3). To coat the sheep erythrocytes with canine thyroglobulin, 0.5 ml of 0.27 M piperazine buffer was added to a 40 cc centrifuge tube. The 0.1 ml of canine thyroglobulin (1.0 mg/ml), 0.1 ml of packed washed erythrocytes, and 0.2 ml of $5 \times 10^{-3}$M chromic chloride ($\text{CrCl}_3$) were added sequentially to the tube. The suspension was agitated gently for 4 to 5 minutes. The reaction was stopped by the addition of 30 ml of saline and the cells were washed 3 times in saline by centrifuging at 1250-1350 rpm for 8 minutes. Sheep erythrocytes were used at a cell concentration of 0.66%. The test was performed with microtiter "V" bottom plates.

**Lymphocyte-blast Transformation Assay (LBT)**

Heparinized blood (10 ml) containing 20 units of preservative-free heparin/ml was collected before the intrathyroidal injection of thyroid
autoantibodies and every week thereafter for 4 weeks. The blood samples were always taken at the same time of the day (between 9 and 10 A.M.).

Blood was diluted with 2 volumes of calcium and magnesium ion-free phosphate-buffered saline solution (TC-PBS) at pH 7.5. Lymphocytes were isolated by centrifugation over commercial lymphocyte separation medium (LSM, Litton Bionetics, Kensington, MD) (specific density 1.077 - 1.080 g/cm³). The preparation was centrifuged at room temperature for 40 minutes at 1800 rpm. The mononuclear leukocytes at the plasma-LSM interface were carefully aspirated and suspended in an equal volume of TC-PBS. Cells were washed 3 times in TC-PBS (1000 rpm for 10 minutes) and were suspended in Eagle's minimum essential medium with Spinner salts (EMEM-S), containing 1% antibiotics (penicillin, 2000 U/ml; streptomycin, 10 mg/ml; and nystatin, 5000 U/ml). The final preparation was adjusted to 10⁶ mononuclear cells/ml with media (EMEM-S) containing 20% heat-inactivated homologous canine serum. The same pool of canine serum was used throughout the experiment.

The LBT assay was performed according to the method described for the dog by Krakowka et al. (18). The peripheral blood mononuclear cells were cultured in triplicate in flat bottom microtiter plates at 10⁵ viable cells per well with an equal volume of phytohemagglutinin-P diluted 3 µg/ml in media or an equal volume of growth media. The cultures were incubated 52 hours at 37°C in a humidified 5% CO₂ incubator. Tritiated thymidine (0.5 µCi) was added to each culture well and incubated for another 18 hours. Subsequently, cells were collected and washed with a multiple automatic sample harvester. Radioactivity incorporated into newly synthesized cellular nucleic acid was trapped on glass filter paper which was dropped in scintillation vials filled with
a toluene-base cocktail (Permablend II, Packard Instruments Co., Downers Grove, IL). Counts per minute were determined for each culture in a liquid beta scintillation counter (Beckman Model LS 8100). The degree of stimulation of lymphocytes by phytohemagglutinin-P was measured in counts per minute (CPM). A stimulation index (SI) was calculated by dividing stimulated counts by unstimulated counts (Table 1).

Lymphocyte blast transformation (LBT) assay also was performed using peripheral blood lymphocytes from clinically normal dogs incubated with mitogen and canine serum containing thyroid autoantibodies (1:1280). In the control group, peripheral blood lymphocytes were incubated with mitogen and a pool of normal canine serum.

Statistical Analysis

Data from the LBT and thyroid hormone assays were analysed statistically by the one-way analysis of variance and by the less significant difference contrast (LSD). This is a Student's "T" test which takes in account the multiple comparison between groups (19).

Light and Electron Microscopy

A longitudinal section of each thyroid lobe through the injection site was fixed in Bouin's fluid, cut at 4 μ, and stained with hematoxylin and eosin. Ten to 20 one mm cubes from an area in close proximity to the injection site and 2 other areas between the injection site and pole of each thyroid lobe were collected for electron microscopy, fixed in ice cold 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, and post-fixed with 1.33% osmium tetroxide in s-collidine at pH 7.2. Tissue blocks were dehydrated in ascending concentrations
of ethanol, transferred to propylene oxide, and embedded in Epon 812. One micron, thick sections were cut from each block and stained with toluidine blue for light microscopic evaluation and selection of the most appropriate area for sectioning. Thin sections were cut at 600 to 800 Å and stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope.

RESULTS

Light Microscopy of Thyroid

Autoantibody - 1 Week. Thyroid glands of dogs 1 week after the injection of thyroglobulin autoantibodies were composed of thyroid follicles containing densely eosinophilic colloid with occasional degenerate cells. They were lined by cuboidal to low columnar cells with occasional endocytotic vacuoles, especially in smaller follicles. There was no accumulation of inflammatory cells at one week.

Autoantibody - 1 Month. Thyroids 1 month following intrathyroidal injection of thyroglobulin antibodies had focal accumulations of lymphocytes and macrophages in 4 of 5 dogs (Figures 1, 2, 3). Isolated degenerate follicular cells with pyknotic nuclei and a densely eosinophilic cytoplasm were present in the follicular wall, colloid, and interstitium. The remaining thyroid follicles were lined by cuboidal to low columnar follicular cells and contained eosinophilic colloid with occasional endocytotic vacuoles.

Normal Serum - 1 Week. One week after the intrathyroidal injection of normal canine serum, the thyroid gland was composed of follicles of varying size lined by cuboidal to low columnar cells. They contained densely
eosinophilic colloid with occasional endocytotic vacuoles near the luminal aspect of follicular cells. Occasional degenerate cells with pyknotic nuclei were admixed with erythrocytes in the colloid of scattered thyroid follicles.

**Normal Serum - 1 Month.** At 1 month (3 weeks following hemithyroidectomy) the follicles were lined by columnar cells with more frequent endocytotic vacuoles near the interface between colloid and the apical surface of follicular cells (Figure 4). The lumen of occasional follicles contained degenerate follicular cells with pyknotic nuclei.

**Electron Microscopy of Thyroid**

**Autoantibody - 1 Week.** Thyroid glands injected with thyroglobulin autoantibodies at 1 week were characterized by a mild interstitial infiltration of lymphocytes and macrophages. Lymphocytes with cytoplasmic projections were attached to convoluted and occasionally reduplicated basement membrane that contained cell debris (Figure 5). The adjacent follicular cells had swollen mitochondria and numerous electron-dense lysosomal bodies. Isolated degenerate follicular cells with markedly dilated profiles of rough endoplasmic reticulum, swollen mitochondria with disrupted cristae, colloid droplets, and irregularly clumped nuclear chromatin were present in the follicular wall (Figure 6). The apical plasma membrane appeared to be focally disrupted and there were large irregular areas of colloid accumulation in the adjacent part of the degenerate follicular cell. The unaffected thyroid follicles were lined by cuboidal to columnar cells with a well developed rough endoplasmic reticulum with occasional pseudopodia on the apical surface and intracytoplasmic microfollicles.
Autoantibody - 1 Month. Thyroid glands at 1 month following injection of autoantibody (3 weeks after the hemithyroidectomy) had multifocal interstitial accumulations of lymphocytes and macrophages. Many inflammatory cells were present in vascular lumens. Isolated degenerate follicular cells with dilated profiles of rough endoplasmic reticulum, irregularly clumped nuclear chromatin, and large accumulations of colloid-like material in the apical cytoplasm were present in the wall of scattered follicles (Figure 7). Adjacent follicular cells appeared to be unaffected. There was a mild increase of collagen fibers in the interfollicular area compared to dogs injected with normal serum.

Normal Serum - 1 Week. Thyroid glands 1 week after the injection of canine serum without thyroid autoantibodies were normal except for an occasional lymphocyte, macrophage, and plasma cell in the interstitium and follicular wall. Thyroid follicles were lined either by cuboidal follicular cells with a small cytoplasmic area and short microvilli or by low columnar cells with more abundant rough endoplasmic reticulum and longer microvilli on the luminal surface.

Normal Serum - 1 Month. At 1 month (3 weeks post-hemithyroidectomy), thyroid follicles were lined by columnar epithelial cells with dilated profiles of rough endoplasmic reticulum, a prominent Golgi apparatus, and long microvilli (Figure 8). Other follicular cells were tall columnar and had many colloid droplets in the apical part of the cytoplasm. Infrequent microfollicles were formed by invagination of the apical surface of the follicular cell. Small thyroid follicles were more numerous than at 1 week but lymphocytes and macrophages were observed less frequently in the interstitium.
Immunologic Studies

The CCH test used to determine the presence of thyroglobulin autoantibodies in serum was consistently negative in the 2 groups of experimental dogs. A positive control serum (titer of 1:1280) obtained from a rabbit immunized with canine thyroid extract in complete Freund adjuvant was consistently positive indicating that the CCH assay was capable of detecting antibody activity in the experimental sera.

Although the degree of lymphocyte stimulation in the control group varied during the experiment, these changes were not significantly different from baseline values. Phytomitogen-induced LBT of dogs in group 2 demonstrated a significant (p<0.05) increase in mitogenic response compared to baseline on days 14, 21 and 28. The LBT response of dogs injected with thyroglobulin autoantibodies (group 2) was significantly increased (p<0.05) compared to the control group on days 14 and 21. The percent increase in LBT above baseline was used to illustrate the effects of an intrathyroidal injection of either normal or hyperimmune canine serum on the incorporation of tritiated thymidine by peripheral blood mononuclear cells (Figure 9). The stimulatory effect of the intrathyroidal injection of thyroid autoantibodies on the mitotic activity of the peripheral blood lymphocytes was maximal on day 14 and was more than 5 times the baseline response. This peak increase was followed by a decrease in lymphocyte-blast transformation to approximately 2 times the baseline on day 28.

The response of normal peripheral blood lymphocytes when incubated with mitogen and canine serum containing thyroid autoantibodies (CPM=4656 ± 889) was not significantly different from normal lymphocytes incubated with canine serum without thyroid autoantibodies (CPM=3172 ± 828).
Thyroid Hormone Levels

Circulating levels of thyroid hormones did not change significantly between the baseline (day 0) and days 7, 14, 21 and 28 after either the intrathyroidal injection of a similar volume of normal canine serum (group 1) or thyroid autoantibodies (group 2). A decrease in circulating thyroid hormone levels was observed on day 7 in the control group followed by a numerical increase above baseline on days 14, 21 and 28 of the experiment (Table 2).

DISCUSSION

The results of this investigation demonstrated that experimental dogs injected intrathyroidally with thyroglobulin autoantibodies develop multifocal lymphocytic thyroiditis with accumulations of macrophages at 1 month. The development of thyroiditis was associated with an increased incorporation of tritiated thymidine by the peripheral blood mononuclear cells but circulating thyroid hormone levels and thyroglobulin autoantibody titers did not change significantly from baseline values. Control dogs injected with serum without thyroglobulin autoantibodies did not develop lymphocytic thyroiditis and the mitogenic response of peripheral lymphocytes was similar to baseline values. The accumulation of inflammatory cells with the formation of detectable foci by light microscopy and the presence of inflammatory cells in association with isolated or groups of degenerate follicular cells were observed only in thyroid glands collected one month after the intrathyroidal injection of thyroid autoantibodies. Thyroid lesions in experimental dogs were similar to the focal autoimmune lymphocytic thyroiditis reported in laboratory beagles (20).
Current evidence suggests that autoimmune thyroiditis is an antibody-mediated, rather than a cell-mediated, immunopathologic process. In the Obese strain (OS) of white Leghorn chickens with naturally occurring lymphocytic thyroiditis, thymectomy accelerated the development of autoimmune thyroiditis (21) whereas bursectomy on the day of hatching prevented the development of the disease (22). It was concluded from these studies that thymus-derived cells failed to suppress the autoimmune reaction and resulted in the development of thyroiditis. Thymectomized normal chickens (Cornell strain, CS) failed to develop thyroiditis after transfer of serum containing thyroid autoantibodies from the OS chickens (23). The differential susceptibility to thyroglobulin autoantibodies between the 2 strains of chickens was explained by normal suppressor activity, insufficient number of killer cells, thyroid-specific B or T cells in CS chickens, and by genetic differences between thyroid glands.

Thyroid autoantibodies when injected into the gland of experimental dogs may have interacted with follicular cells and initiated cellular destruction via complement fixation or through an in vivo antibody-dependent cell-mediated cytotoxicity (ADCC). Thyroiditis has been reported to be as severe in animals depleted of C3 or congenitally lacking C5, as in intact animals indicating that antibodies in collaboration with complement were not required in the pathogenesis of autoimmune thyroiditis (24). Thyroid autoantibodies have been demonstrated to be produced within the thyroid gland in naturally occurring autoimmune thyroiditis (25). K cells with receptors for the Fc region of IgG but without conventional B or T lymphocyte markers (26) are believed to be the effector cell in ADCC and have been reported to accumulate in thyroids with lymphocytic thyroiditis induced by immunization with complete Freund's
adjuvant (27). During the reaction between lymphoid cells and follicular cells, lymphokines and other factors appear to be released which may influence the function of peripheral blood lymphocytes. The increased proliferative response of peripheral lymphocytes to mitogen in the LBT assay that was observed in our experimental dogs may be explained by this mechanism. The temporal sequence of lymphocyte transformation to thyroid antigen during the development of experimental thyroiditis induced with thyroid extract in complete Freund's adjuvant (28) was similar to changes observed in the LBT assay of experimental dogs.

Any direct effects of thyroglobulin autoantibodies on the function of lymphocytes seem unlikely because 1) thyroglobulin autoantibodies were not detected in the circulation by the CCH test and 2) serum containing thyroid autoantibodies when incubated with normal lymphocytes and mitogen did not result in a significant change in mitogenic activity.

The intrathyroidal injection of thyroid autoantibodies did produce a physical disruption of scattered thyroid follicles with leakage of colloid into the interstitium. Thyroid autoantibodies may have bound to the antigens present in the colloid and formed immune complexes. An interstitial thyroiditis has been induced in mice by immunization with heterologous thyroglobulin (29). Thyroglobulin, IgG and C3 were identified by immunofluorescence and were associated with the numerous polymorphonuclear cells and scattered mononuclear cells that infiltrated the thyroid. Electron-dense deposits were present between the follicular cell and basement membrane. The thyroiditis induced in our dogs was characterized by an infiltration predominately of lymphocytes and macrophages. Electron-dense deposits resembling antigen-antibody complexes
were not identified in the basement membrane. Thyroid lesions observed in experimental dogs were not as severe as the lesions reported in pet dogs with naturally occurring lymphocytic thyroiditis (2). The thyroid lesions remained focal after 1 month because autoantibodies appeared to be degraded or used immediately in the gland. It may be necessary to give repeated intraglandular or parenteral injections of thyroid autoantibodies in order to produce a progressive thyroiditis as has been demonstrated in experimental allergic thyroiditis in dogs (30).

The morphology of the thyroid lesions (2) and frequent occurrence of circulating thyroglobulin autoantibodies (3) in pet dogs with clinical hypothyroidism suggested that naturally occurring lymphocytic thyroiditis was immune-mediated. Thyroid lesions similar to naturally occurring autoimmune thyroiditis in dogs were induced by a local thyroidal graft-versus-host reaction (31). The lesions observed in the thyroid lobe which was not injected with immunocompetent cells appeared to develop from the formation of thyroid antibodies in the gland by migrating host lymphocytes. The results of the present investigation demonstrated that the intrathyroidal injection of thyroglobulin autoantibodies obtained from a dog with naturally occurring lymphocytic thyroiditis also could induce lesions similar to naturally occurring lymphocytic thyroiditis and suggests that thyroid autoantibodies play an important role in the pathogenesis of thyroiditis in dogs.

**SUMMARY**

Multifocal infiltration of lymphocytes and macrophages were present in the thyroid gland of dogs one month after an intrathyroidal injection (0.5 ml) of
serum containing thyroglobulin autoantibodies (titer 1:1280 by the chromic chloride hemagglutination test). The development of thyroiditis was associated with a gradual increase in the incorporation of tritiated thymidine by the peripheral blood mononuclear cells. There was no significant alteration in circulating thyroid hormone levels or thyroglobulin autoantibody titer during the experiment. Control dogs injected with a similar volume of canine serum without thyroglobulin antibodies did not have an inflammatory reaction in the thyroid gland and the lymphocyte-blast transformation assay did not change compared to baseline values.

The findings that an intrathyroidal injection of canine thyroglobulin autoantibodies could induce lesions similar to naturally occurring lymphocytic thyroiditis in dogs suggests that thyroid autoantibodies play an important role in the pathogenesis of immune-mediated thyroiditis.
Table 4.1. Lymphocyte-Blast Transformation (LBT) in Response to Phytohemagglutinin-P* Expressed as Mean Counts/Min (CPM) and Stimulation Index (SI) in Dogs (5/Group) Injected with Either Normal or Canine Serum Containing Thyroid Autoantibodies.

<table>
<thead>
<tr>
<th>Group</th>
<th>Days After Intrathyroidal Injection</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CPM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1768</td>
<td>1910</td>
<td>2326</td>
<td>2732</td>
<td>2518</td>
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<td></td>
<td>+69</td>
<td>+87</td>
<td>+146</td>
<td>+434</td>
<td>+364</td>
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<tr>
<td>Tg-Ab**</td>
<td>2383</td>
<td>4111</td>
<td>9690‡‡</td>
<td>8352‡‡</td>
<td>7375‡</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+699</td>
<td>+478</td>
<td>+1274</td>
<td>+745</td>
<td>+1500</td>
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</tr>
<tr>
<td></td>
<td>SI***</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>62.5</td>
<td>62.3</td>
<td>78.6</td>
<td>52.7</td>
<td>51.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+8.8</td>
<td>+13.8</td>
<td>+13.8</td>
<td>+16.2</td>
<td>+8.0</td>
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<tr>
<td>Tg-Ab**</td>
<td>71.9</td>
<td>82.0</td>
<td>107.4</td>
<td>170.5</td>
<td>162.1</td>
<td></td>
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<tr>
<td></td>
<td>+21.9</td>
<td>+18.7</td>
<td>+34.9</td>
<td>+60.9</td>
<td>+124.4</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± standard error.
** Tg-Ab = group of dogs injected with hyperimmune canine serum.
*** Stimulated CPM/unstimulated CPM.
* Significantly different from the baseline response (p<0.05).
** Significantly different from the control group (p<0.05).
Table 4.2. Serum Thyroxine (T₄) and Triiodothyronine (T₃)* Before and After The Intrathyroidal Injection of Dogs (5/Group) with Either Normal or Canine Serum Containing Thyroid Autoantibodies.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Groups</th>
<th>Days After Intrathyroidal Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>T₃ (ng/dl)</td>
<td>Control</td>
<td>37.6</td>
</tr>
<tr>
<td></td>
<td>Tg-Ab⁺</td>
<td>51.5</td>
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<tr>
<td></td>
<td></td>
<td>+6.1</td>
</tr>
<tr>
<td></td>
<td>Tg-Ab⁺</td>
<td>+13.9</td>
</tr>
<tr>
<td>T₄ (μg/dl)</td>
<td>Control</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>Tg-Ab⁺</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+0.17</td>
</tr>
<tr>
<td></td>
<td>Tg-Ab⁺</td>
<td>+0.14</td>
</tr>
</tbody>
</table>

* Mean ± standard error.
⁺ Tg-AB = Group 2: dogs injected with hyperimmune canine serum.
Figure 4.1. Thyroid gland injected with thyroglobulin autoantibodies at one month. Focal interstitial accumulation of lymphocytes and macrophages surrounding a small vessel (V). Thyroid follicles are lined by cuboidal to low columnar cells with occasional vacuoles (arrow) in the colloid. (H&E, X 315)
Figure 4.2. Thyroid gland injected with thyroglobulin autoantibodies at one month. Note the extensive interstitial infiltration of lymphocytes and macrophages near small follicles (F) containing little colloid. (H&E, X 315)
Figure 4.3. Thyroid gland injected with thyroglobulin autoantibodies at one month. There is extensive interstitial infiltration of lymphocytes and macrophages around small follicles (F) and adjacent groups of C-cells (P). (H&E, X 315)
Figure 4.4. Thyroid gland injected with normal canine serum at one month. Thyroid follicles (F) are lined by cuboidal to low columnar cells and had occasional vacuoles in the colloid (arrow). (H&E, X 315)
Figure 4.5. Thyroid gland injected with thyroglobulin autoantibodies at one week. A cytoplasmic projection of a lymphocyte (L) is attached to an irregularly convoluted basement membrane (B). The adjacent follicular cell has swollen mitochondria with disrupted cristae and numerous lysosomal bodies in the cytoplasm (M). A degenerate cell (C) is present in the interstitium. X 3400.
Figure 4.6. Thyroid gland injected with thyroglobulin autoantibodies at one week. An isolated degenerate follicular cell with dilated profiles of rough endoplasmic reticulum (E), irregularly clumped nuclear chromatin (N), and colloid droplets (C) in the cytoplasm is present in the follicular wall. The adjacent follicular cells appeared normal (F). The luminal surface of the follicular cell appears to be disrupted (arrows). X 3400.
Figure 4.7. Thyroid gland injected with thyroid antibodies at one month. Isolated degenerate follicular cells with irregularly clumped nuclear chromatin (N), profiles of rough endoplasmic reticulum (E), and irregular accumulations of colloid-like material (C) were present in the follicular wall. The luminal surface of the follicular cell appears to be disrupted (arrows). Adjacent follicular cells (F) are unaffected. Numerous collagen fibers (CF) are present in the interfollicular space. X 4900
Figure 4.8. Thyroid gland injected with normal canine serum at one month. The thyroid follicle is lined by cuboidal follicular cells with profiles of rough endoplasmic reticulum (E), mitochondria, microvilli (arrows) on the luminal surface, and myelin bodies (B) in the cytoplasm. Occasional collagen fibers are present in the interfollicular space. X 3400.
Figure 4.9. Lymphocyte-blast transformation in response to phytohemagglutinin-P expressed as percent of baseline response in dogs injected with normal or hyperimmune canine serum containing thyroid autoantibodies (1:1280).
LYMPHOCYTE BLAST TRANSFORMATION

% INCREASE (CPM) ABOVE BASELINE RESPONSE

HEMITHYROIDECTOMY

THYROID INJECTED WITH NORMAL SERUM
THYROID INJECTED WITH THYROID AUTOANTIBODIES
N = 5 DOGS / GROUP

Fig. 9
BIBLIOGRAPHY

CHAPTER I


CHAPTER II


CHAPTER III


CHAPTER IV


