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INVESTIGATION OF AUTOANTIBODIES TO THYROGLOBULIN, THYROXINE, TRIIODOTHYRONINE AND THYROID PEROXIDASE IN DOGS

By

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ABSTRACT

INVESTIGATION OF AUTOANTIBODIES TO THYROGLOBULIN, THYROXINE, TRIIODOTHYRONINE AND THYROID PEROXIDASE IN DOGS

By

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Assays were developed to detect autoantibodies to thyroglobulin (Tg), thyroxine (T_4), triiodothyronine (T_3), and thyroid peroxidase (TPO) in dogs. An enzyme-linked immunosorbent assay (ELISA) was used to detect autoantibodies to Tg. Autoantibodies to T_4 and T_3 were detected using radioisotope labeled T_4 and T_3 in an agarose gel electrophoresis assay. An initial study of 119 canine sera from dogs with clinical signs of hypothyroidism including alopecia, lethargy and obesity found 58 (48.7%) had autoantibodies to Tg, T_4, or T_3. The presence of Tg autoantibodies was seen more frequently ($P<0.05$) in samples of low serum T_4 concentrations. In a second study of a group of 135 Golden Retrievers, with no clinical signs of hypothyroidism, 16.3% of the sera tested had autoantibodies to one of the 3 antigens. Forty-two Golden Retrievers related to a dog with autoantibodies from the initial study population were tested for the presence of autoantibodies to Tg, T_4, or T_3. Of these dogs, 6 (14.3%) had autoantibodies. Autoantibodies to Tg, T_4, or T_3 were present in significantly higher frequency in Golden Retrievers 1 year of age or younger ($P<0.05$).

Canine TPO was isolated to determine whether autoantibodies to TPO occur in canine hypothyroidism, as is the case in human autoimmune thyroiditis. The enzyme was solubilized from the thyroid using a deoxycholate-trypsin digestion, ammonium sulfate precipitation, differential centrifugation and DEAE sephadex chromatography. A monoclonal antibody to canine Tg coupled to a immunoaffinity column was used to eliminate the contaminating Tg from the TPO preparation. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions indicated protein bands at 107 and 47 kDa. SDS-PAGE, under reducing conditions eliminated the 107 kDa band which was replaced with components at 60, 45, and 39 kDa. A Western immunoblot assay was developed using the isolated TPO. Fifty canine sera from dogs with autoantibodies to Tg, T_4, or T_3 were screened by Western blot for evidence of autoantibodies to TPO. No antibodies were detected in these sera suggesting a different pathogenesis for canine autoimmune thyroid disease than that hypothesized for human autoimmune thyroiditis.

Dedicated with love to

my husband, Brad, and

my children, Stacy and Paul.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Hypothyroidism in dogs is a frequently diagnosed (or misdiagnosed) disorder. Because the clinical symptoms; alopecia, lethargy, obesity and reproductive failure are non-specific, hypothyroidism is difficult to differentiate from other dermatologic, neurologic or reproductive disorders.

Currently, the diagnosis of canine hypothyroidism is based on the measurement of thyroid hormone concentrations in the serum. However, thyroid hormone concentrations are frequently not affected until the later stages of thyroid disease and as such, are of limited value in diagnosing early stages of the disease. Compounding the diagnostic problem are the many factors that affect thyroid hormone concentrations. Furthermore, many drugs and metabolic changes lower thyroid serum concentrations while the dog is physiologically euthyroid. In other words, the dog is not hypothyroid even though the serum thyroid hormone concentrations are low.

Primary diseases of the thyroid are the most common cause of hypothyroidism in dogs. Idiopathic thyroid atrophy and lymphocytic thyroiditis account for the majority of canine primary autoimmune thyroid diseases.[1] Idiopathic thyroid atrophy is characterized by loss of thyroid parenchyma with adipose and connective tissue replacement. The cause of this disorder is unknown and there has been speculation that this is actually an end-stage result of

lymphocytic thyroiditis. Lymphocytic thyroiditis is characterized by the infiltration of the thyroid gland by mononuclear cells, lymphocytes being the most predominate type which results in the destruction of the thyroid follicles and formation of lymphoid follicles. Fibrosis of the thyroid parenchyma is seen in advanced cases with minimal inflammatory cell infiltrate. In a colony of laboratory beagles, focal lymphocytic thyroiditis was observed with no significant changes in thyroid function or accompanying clinical signs.[2] However in pet dogs, lesions tend to be more diffuse and extensive with clinical signs of hypothyroidism occurring as the thyroid gland is progressively destroyed.[3]

While the initiating cause of autoimmune lymphocytic thyroiditis is unknown, both the cellular and humoral immune systems play a role in the destruction of the thyroid gland. The formation of autoantibodies to various thyroid antigens is indicative of an alteration in the immune system. Autoantibodies to thyroglobulin (Tg), thyroxine (T_4), triiodothyronine (T_3), and the microsomal antigen which has been identified as thyroid peroxidase (TPO) in humans have been identified in dogs and human beings with thyroid disease.[4-6] In studies of humans with Hashimoto's thyroiditis, the presence of immune complexes composed of Tg and anti-Tg antibodies have been found on the thyroid follicular basement membrane. The formation of these complexes may lead to thyroid damage by fixation of complement or by activation

of killer cell activity.[6] Increased activity of cytotoxic T cells and/or the cytotoxic effects of lymphokines released by activated T cells responding to thyroid antigens are potential cell mediated immune mechanisms that damage the thyroid.[7]

The detection of autoantibodies to the various thyroid antigens is an important tool in the diagnosis of thyroid disease in human beings. Greater than 90% of patients with Hashimoto's thyroiditis have autoantibodies to TPO. Autoantibodies to thyroid antigens have also been studied in the dog.[3-5] The incidence of these autoantibodies have varied with the type of assay used for their detection and the population of dogs tested. The incidence of autoantibodies to Tg in various populations of dogs has ranged from 27 to 73 percent. Autoantibodies to T_4 and T_3 were found in 0.2 percent of the canine sera samples submitted to Auburn University for the assay of thyroid hormone concentrations.[8] In addition, microsomal autoantibodies were detected in 4 to 86 percent of various studies of hypothyroid dogs. Because of the wide variation in ranges of autoantibodies detected many questions remain as to the role and importance of these autoantibodies in dogs with lymphocytic thyroiditis and clinical hypothyroidism.

Previous studies in dogs employed complement fixation or indirect immunofluorescence assays to identify microsomal autoantibodies.[4,5] Because in human beings, the microsomal autoantibodies had been demonstrated to fix complement,

complement fixation using a crude thyroid extract was performed. Indirect immunofluorescence was also used in the dog to investigate thyroid autoantibodies. It has since been documented in human beings that the microsomal antigen is TPO.

This study looked at the various thyroid antigens and autoantibodies in the dog and their relationship to each other and to the clinical signs of hypothyroidism. The development of assays for these various autoantibodies may help diagnose autoimmune lymphocytic thyroiditis in the dog. Looking at autoantibodies to the various thyroid antigens may improve our ability to determine the presence or absence of thyroid disease with improved accuracy. In addition to improved diagnostic assays, determination of the thyroid antigens that induce thyroid autoantibody formation may be beneficial to investigating the pathogenesis of thyroid disease and answer the question of what is responsible for the body no longer recognizing the thyroid as a part of itself.

The specific objectives of this study were to develop assays to detect autoantibodies to canine Tg, T_4, T_3 and the microsomal antigen using purified canine TPO. After these assays had been developed, the relationship between thyroid autoantibodies and the clinical signs of hypothyroidism in dogs and their relationship to serum thyroid hormone concentrations were investigated.

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LITERATURE REVIEW

Thyroid Anatomy

The thyroid gland was named by the Greeks because of the resemblance the human thyroid gland has to a shield. In the dog, the thyroid gland is not shield-like, but rather is 2 separate lobes lying dorsal-lateral to the first five to eight tracheal rings. An isthmus, connecting the two lobes is occasionally found on the ventral surface of the trachea. In most dogs the cranial pole of the right lobe lies opposite the first tracheal ring or the caudal border of the cricoid cartilage of the larynx. The left lobe usually lies one to three tracheal rings caudal to the right lobe.

The thyroid glands lie in fascia which is attached to the overlying musculature and the trachea. The glands in dogs are not as tightly attached to the trachea as they are in humans. The size of the lobes are variable, the mass of one of the lobes occasionally being as much as 50% larger than the other. Generally, younger subjects have larger thyroid glands which decrease in size with age. The glands have been reported to be larger in females than in males, and may have a transitory enlargement during estrus and pregnancy.[1] A thyroid lobe in a 10 to 15 kg dog is approximately 2 X 1.5 X 0.5 cm.[2] The normal thyroid gland ranges from 40 to 400 mg/kg body

weight. The absolute weight of the thyroid glands may be increased in areas of iodine deficiency, where weights of 80 to 1600 mg/kg have been reported.[3]

The dorsal edge of the thyroid glands lie in close proximity to the carotid sheath carrying the carotid artery, vagosympathetic nerve trunk, internal jugular vein and the recurrent laryngeal nerve. The lobes lie in the fascial compartment composed laterally of the deep lamina of the middle cervical fascia of the sternocephalicus muscles, and medially of the prevertebral fascia which communicates with the thoracic cavity.

There is a richer blood supply to the thyroid glands then almost any other organ in the body. The cranial thyroid artery arising from the common carotid artery is the principal blood supply to the thyroid glands. Occasionally a caudal thyroid artery originating from the brachycephalic artery is also present. Beneath the fibrous capsule of the thyroid gland, the blood vessels form a vascular plexus. This plexus gives rise to a capillary network which surrounds each follicle, the capillary endothelium being contiguous with the follicular epithelium. The principal venous return from the thyroid gland is the caudal thyroid vein which leaves the caudal end of the lobe and joins the internal jugular vein.

The thyroid glands are innervated by fibers from the sympathetic component of the cranial laryngeal nerve. There are also parasympathetic nerve fibers from the vagus. Hormone

secretion is indirectly influenced by blood flow through the gland, which is controlled by nerve plexuses in the walls of the arteries. Stimulation of the thyroid nerve slows the flow of blood through the gland, whereas stimulation of the homolateral vagus accelerates the blood flow. However, the most important factor in the control of thyroid hormone secretion is the balance maintained between the thyroid hormones and the thyrotrophic hormones of the anterior hypophysis.

Microscopically, the thyroid gland has two capsules. The outer capsule is continuous with the cervical fascia. The inner capsule is composed of fibroelastic tissue from which septa extend into the thyroid parenchyma. The septa provide the internal support for innervation, blood supply and lymphatics and separate the thyroid into lobules. Because the septa are not connected, the thyroid gland is not actually lobulated, but pseudolobulated.

The thyroid gland consist of follicles which are the structural and functional unit of the thyroid. A follicle consists of a single layer of epithelial cells enclosing a cavity, which is filled with colloid. The follicles are surrounded by a thin basement lamina and a network of capillaries and nerves. The shape of the follicular cells varies from follicle to follicle and with the activity of the thyroid gland. In general, cuboidal cells are characteristic of hypoactive follicles and columnar cells of hyperactive

follicles. Follicle diameter ranges from 30 to 160 microns, with epithelial height ranging from 8 to 20 microns.[4]

Thyroid follicular cells, when examined with an electron microscope, are similar to other secretory cells, but have several unique features. Numerous microvilli extend into the colloid from the apical surface of the follicular cell. It is at or near this surface of the cell that iodination, exocytosis, and the initial phase of hormone secretion occurs.[6] The nucleus of the follicular cell is located in the basal portion of the cell and has with no distinctive features, but is poor in heterochromatin, and has 1 or more nucleoli. The cytoplasm is laden with extensive endoplasmic reticulum with associated polysomes containing the precursor of thyroglobulin (Tg). The Golgi apparatus is located apically in the cell and resembles other secretory cells. It is in the Golgi complex that the peripheral monosaccharides are incorporated into the carbohydrate units of the Tg molecule.

In addition to the follicle cells, the thyroid glands contain parafollicular or C cells that produce the hormone calcitonin. These cells are found either in the follicle wall or in the interfollicular spaces.[5]

Usually there are 2 parathyroid glands associated with each thyroid lobe. The external parathyroid lies in the fascia at the cranial pole of the thyroid. Internally, the parathyroid lies beneath the thyroid capsule on the medial

aspect of the lobe; in some cases it may be embedded in the thyroid parenchyma. The internal parathyroids cannot be surgically separated from the thyroid glands. The location of the parathyroids is variable in each individual dog and may vary between the right and left lobes.

Thyroid Physiology

The physiological processes of the thyroid gland are: synthesis in the follicular cells of the specific thyroid glycoprotein, Tg and it's secretion into the follicular lumen; iodination of Tg, including the formation of hormones; and the storage of Tg in the follicular lumen, reabsorption of Tg into the follicular cell, degradation of Tg and release of hormones into the adjacent vasculature.

The follicle is the smallest functional unit of the thyroid gland. In the active follicular cells, the rough surfaced endoplasmic reticulum (RER) is a prominent component. Most of the protein synthesis occurring in the RER is directed toward the production of Tg. The synthesis of the peptide chain of Tg occurs in the polysomes attached to the RER membranes as the Tg is transported across the RER membrane.[7,8]

The Tg is transported via transitory vesicles to the Golgi apparatus and involves energy dependent membrane fissions and fusions.[9] It is in the Golgi apparatus that the majority of peripheral monosaccharides are incorporated into

the carbohydrate units of the Tg molecules.[10] From the Golgi apparatus, Tg is transported to the apical surface in vesicles. The vesicles fuse with the apical surface by exocytosis and empty their contents into the follicular lumen.[11] Upon stimulation with thyroid stimulating hormone (TSH), apical microvilli extend into the colloid in the follicle lumen and fuse, leading to endocytotic uptake of Tg and formation of colloid droplets in the cytoplasm. These colloid droplets fuse with lysosomes to form phagolysomes in which acid proteases and peptidases degrade the Tg into iodothyronines including thyroxine (T_4) and triiodothyronine (T_3) the active thyroid hormones. The thyroid hormones, T4 and T3, are released from the phagolysosomes at the base of the cell into blood vessels while the remaining products of Tg breakdown wo form new thyroid hormone molecules.

Iodide transport occurs with the pumping of iodide from the plasma into the thyroid, where it is concentrated, oxidized and used in thyroid hormone synthesis. Iodide (I^-) is immediately oxidized within the thyroid to the organic form (I) by H_2O_2 and O_2. The transport of iodide is temperature dependent and requires an intact cellular membrane but not an intact follicule as isolated thyroid cells can concentrate iodide.[12] The iodide concentrating mechanism is located in the basal membrane, requires external K^+ and appears to be a Na^+ transport mechanism involving Na^+-K^+ ATPase as demonstrated by inhibition by ouabain.[13] Iodide transport is an active process

as the iodide is transported against an electrochemical concentration gradient. Thyroid stimulating hormone (TSH) plays a major role in iodide accumulation. With an increase in TSH, there is an increase in the influx of iodide modulated by cAMP.[14]

The transport of iodide across the plasma membrane involves phospholipid fractions which complex the iodide and make it more soluble. Thyroid phospholipid binding is similar to an enzyme reaction in regard to saturation kinetics, structural specificity, and the competition by other anions. Other proteins, especially albumin have been suggested as carriers for iodide to the thyroid. In addition to iodide ions, the thyroid can concentrate other halides and complex anions which exhibit competitive inhibition of iodide uptake.[12]

The activity of the iodide transport system in the thyroid glands varies inversely with the circulating levels of iodide. As a consequence of this autoregulation, plasma iodine concentrations influence thyroid hormone synthesis by altering the iodide transport in the thyroid.[15] However, normal individuals exposed to chronic iodide excess escape the autoregulation as the iodide transport system adapts to limit intracellular iodide. As a result, the intra-thyroidal iodide concentration falls below the level necessary to inhibit iodotyrosine formation.[16] Since cAMP production is inhibited by iodides, the inhibition of adenylate cyclase has been suggested as the escape mechanism.[17]

Thyroglobulin (Tg)

Thyroglobulin is the iodinated protein which is the biosynthetic precursor of the thyroid hormones and has an essential role in thyroid hormone homeostasis. Thyroglobulin allows the utilization of iodine to produce a high yield of thyroid hormones. The presence of high and low affinity sites for iodination allows Tg to modulate hormone synthesis in relation to iodine availability. Thyroglobulin allows the storage of thyroid hormones in a non-active form in extracellular space away from metabolic influence.

Structurally Tg is a dimeric glycoprotein with a molecular weight of 660,000 daltons and a sedimentation coefficient of 19S. Examination with an electron microscope reveals 2 symmetric, identical subunits of 330,000 daltons.[18] The amino acid composition of Tg is not unusual and doesn't contain an unusual amount of tyrosine, from which the thyroid hormones, T_4 and T_3 are composed. N-glycosylation of the peptide chain results in two types of oligosaccharide units representing eight to ten percent by weight of the molecule depending on the species.[19,20] After synthesis and glycosylation of the peptide chains, the protein is iodinated and degraded to produce the thyroid hormones.

Thyroglobulin has been found to have specific structural domains involved in thyroid hormone formation. In human beings, a single Tg molecule contains 110 tyrosine residues,

two-thirds of which are on the surface of the molecule, and in vivo a maximum of

twenty are iodinated giving rise to two to five hormonally active iodothyronines.[21] Porcine Tg also contains several T4 forming sites which have different primary structure and different hormone synthesizing capacity than human beings.[22] The same hormonogenic structure can synthesize T4 or T3 depending on the amount of iodination of the thyroglobulin.

Non-iodinated, newly formed Tg dissociates freely into 12S subunits by dilution, alkaline pH or dissociating agents. Prior to iodination, there are ten free sulfhydryl groups on thyroglobulin.[23] As the Tg molecule becomes more iodinated the sulfhydryl groups disappear and it becomes more resistant to dissociation into the 12S subunits. This stability appears to be due to the increasing number of disulfide bridges in the Tg molecule as a result of iodination.[24]

Most iodination of Tg occurs post translation at the microvilli-rich, cell-colloid interface very close to the apical membrane by the enzyme thyroid peroxidase. The Tg then diffuses into the lunenal colloid where some iodination can occur.[25] Colloid is composed almost entirely of Tg with greater than 90% consisting of the 19S Tg. Thyroglobulin breakdown to the thyroid hormones occurs almost exclusively in the follicle cell. The colloid material in the follicle lumen is engulfed by endocytosis forming membrane bound colloid droplets. There are few colloid droplets in normal, resting

thyroid tissue, but their number rapidly increases following the administration of TSH. During endocytosis, a very large surface of the apical plasma membrane is internalized.[26] Exocytotic vesicles containing newly formed Tg may be a source of the membrane engulfed by endocytosis; however the endocytotic and exocytotic membranes are not the same as the endocytotic vesicles are devoid of thyroid peroxidase (TPO) implying that a prior reorganization of the apical membrane is necessary.[11] The mechanism by which the membrane changes is unknown. Enzymes in the lysosomes that hydrolyze Tg include; acid phosphatase, glycoside hydrolases, cathepsin D, several thiol endopeptidases, and several exopeptidases.[21]

Efficient hydrolysis of Tg resulting in release of the thyroid hormones requires that the phagolysomes maintain an acidic pH, which may be driven by an adenosine triphosphate (ATP) pump.[27] Among the iodinated compounds leaving the thyroid gland, T_4, T_3, and iodide are the main constituents. Other minor components include the inactive reverse T_3 (rT_3), trace amounts of monoiodotyrosine (MIT), diiodotyrosine (DIT), diiodothyronines, and Tg. Following the hydrolysis of Tg, the majority of the MIT and DIT, which contain 80% of the Tg iodine, are deiodinated within the follicle cell.[28] The iodide released from the MIT and DIT is reused for hormone synthesis, providing the follicle cell with two to three times the amount of iodide transported from the blood stream.[29]

The mechanism of release of the thyroid hormones into the blood stream following Tg hydrolysis is presently unknown. Under physiological conditions about 90 per cent of the thyroid hormones secreted are T4, of which 75 per cent are deiodinated in peripheral tissues to form the active T3, the remaining T_4 being degraded and excreted.[30]

In some pathological states, the amount of T_3 secretion can increase. Iodine deficiency, for example results in decreased deiodination and increased production of T_3. The high T3 content in Tg is accompanied by a high relative content of T3 in thyroid secretion resulting in a eumetabolic state being maintained.[31] Normally, the T4/T3 ratio in thyroidal secretion is related to the T4/T3 ratio in the Tg hydrolysate, but this latter is not always an exact representation of the iodine content of the gland.[32] The preferred secretion of T3 over T4 under these conditions may also be due in part to increased deiodination of T4 within the follicular cell and a faster secretion of T3 during Tg hydrolysis.[33]

Iodination of Tg is catalyzed by the enzyme thyroid peroxidase (TPO), a membrane-bound, glycosylated hemoprotein with a molecular weight of 100,000 daltons.[34] The amino acid sequence of human TPO has been determined.[35] Thyroid peroxidase has several important functions in the thyroid which include: 1) the oxidation of iodide to a higher oxidation state with H_2O_2 acting as the electron acceptor;

2)binding of the oxidized iodine to tyrosine residues of the Tg molecule; and 3) the coupling of the resulting iodotyrosines.

Because of its particulate nature, TPO has been difficult to purify, and preparations with high specific activity have been isolated only after first releasing the exzyme from the membrane by proteolysis.[36] Although it is likely that the native enzyme is altered by these procedures, the fragment that is isolated is nevertheless extremely active and carries the active site.

As previously discussed, iodide, the form in which iodine enters the thyroid gland, must first be oxidized to a higher oxidation state before it can act as an effective iodinating agent. The exact mechanism of TPO-catalyzed iodination has not been determined at this time, however various theories have been discussed. One theory suggests that that both the I^- and the tyrosyl residue undergo monoelectron oxidation resulting in the formation of free radicals.[37] Another theory suggests a two-electron change producing an intermediate, the iodinium ion, I^+.[38] A third theory proposes that TPO degrades H_2O_2 catalytically in the presence of low concentrations of I^- resulting in the formation of hypoiodite (HIO_3) as the iodinating intermediate.[39] Most investigators suggest that iodination occurs on the enzyme implying that there is a specific site for the acceptor (tyrosine or tyrosyl) Evidence suggests that oxidized TPO has two substrate sites, one

favoring I^- and the other favoring tyrosine. Iodination of tyrosine appears to occur after the tyrosine has been incorporated into thyroglobuin.[40] The iodination of Tg probably occurs on fully aggregated Tg rather than on its subunits.[41]

Without a source of H_2O_2, TPO displays no activity.[39] It appears that H_2O_2 plays an essential role in thyroid hormone formation and it is possible that under some conditions, it could be a rate-limiting step. The pathway for the H_2O_2 generation in the thyroid involves reduced pyridine nucleotides coupled to a flavoprotein cytochrome reductase. This is based on the fact that iodination in the thyroid is stimulated by the addition of NAD(P)H and flavin nucleotides.[42]

Formation of T_4 can occur nonenzymatically by iodination of Tg and other proteins with molecular iodine.[43] The catalytic role for TPO in the coupling reaction has been demonstrated by observing that the amount of labeled T_4 and T_3 increased markedly when the labeled Tg was inclubated with TPO. This increase could not be attributed to a change in the level of iodination in Tg, because the coupling system in contrast to the iodination system contained no added iodide. Moreover, the increase in labeled T_4 and T_3 was accompanied by a corresponding decrease in DIT, indicating conversion of DIT to T_4 and T_3.[44]

Analysis of the Tg gene has been performed in humans, cattle, goats and rats and indicates a split gene of unique

organization composed of approximately 300 kilobases.[45-47] In all species studied there is a predominance of small exons 150-200 base pairs in length homogeneous throughout the gene suggesting this is a pattern under strong selective pressure. A giant 64 kilobase intron is present in the middle of the genes.[45] Hormonogenic sites have been assigned in four exons; 1 at the 5' end of the human gene and 3 at the 3' end of the rat and human gene, although they are in different exons.

Thyroid Regulation

The primary regulatory mechanisms of the thyroid gland are the hypothalamic-pituitary-thyroid control system and an intrathyroidal autoregulatory system. Thyroid stimulating hormone, released from thyrotrophs in the pituitary, stimulates many aspects of thyroid activity including thyroid hormone synthesis and secretion. The secretion of TSH is regulated by two elements: neural control by the hypothalamus and feedback control by thyroid hormones. Three different neurohormones mediate neural control, a stimulating neuropeptide (thyrotropin-releasing hormone [TRH]) and two inhibitory factors, somatostatin and dopamine.[48] These regulating factors are synthesized by neurons in the hypothalamus and reach the anterior pituitary through specialized blood vessels. In addition to regulation by thyroid hormone concentrations, TSH secretion is modified by estrogens, which sensitize the pituitary to TRH, and

glucocorticoids, which reduce pituitary responsiveness to TRH and possibly inhibit TRH secretion.[49-51]

Thyroid hormones act on the pituitary to inhibit TSH secretion.[52] However, uncertainty still exists as to whether or how the brain participates in the feed-back regulation of TSH secretion by modulation of TRH and somatostatin secretion.[48] In tissue cultures of either normal pituitary cells or mouse thyrotropic tumors, both T_4 and T_3 cause a decrease in the spontaneous release of TSH or that induced by TRH.[53] Suppression of TSH secretion by thyroid hormones takes several hours and does not occur if protein synthesis is blocked.[52,53] Initially, thyroid hormones inhibit the release of TSH, but later TSH synthesis is also reduced. Nuclear T_3 and T_4 receptors have been demonstrated on mouse thryotropic cells with T_3 binding having higher avidity than T_4.[53] High doses of thyroid hormones will decrease the population of TRH receptors in the pituitary. Another factor in TSH suppression is the conversion of T_4 to T_3 which also occurs in the pituitary.[54]

TSH binds to specific receptor sites on the plasma membrane of thyroid follicular cells stimulating adenylate cyclase activity, thereby increasing generation of cyclic AMP which appears to be the mediator of most of the hormonal metabolic effect.[55] The adenylate cyclase system in the thyroid resembles that in other tissues as there are three components: the hormone receptor, a guanine nucleotide regulatory protein, and the catalytic activity.[56,57] Cyclic AMP

actives protein kinases, which phosphorylate appropriate substrates, altering their function.[58] Two of the phosphorylated substrates have been identified as the catalytic unit of Na^+, K^+-ATPase and a glycoprotein related to an aggregation-promoting factor.[59] Upon administration of TSH, morphologic alterations occur in the thyroid cell that are associated with enhanced secretion of thyroid hormones. TSH also influences all phases of iodine metabolism from stimulation of the iodide transport to secretion of T_3 and T_4.[60]

In addition to the hypothalamus-pituitary-thyroid axis, the thyroid is regulated by it's requirement for iodine. For a continued flow of thyroid hormones to the tissues, the thyroid has a mechanism that adapts to the iodine available, and the level of thyroid hormones. Administration of excess iodide reduces the formation of cAMP induced by TSH by unknown mechanisms.[61] As previously discussed, excess iodide decreases the iodide transport system and inhibits iodide incorporation into Tg.[62]

Thyroid Hormones

Thyroxine is the primary secretory product of the thyroid gland, however T_3 is the active hormone in metabolic regulation. Thyroid hormones are formed from some of the iodotyrosine residues that are present in the thyroglobulin molecule. In studies of the concentrations of T_4 and T_3 from Tg metabolism, it has been demonstrated T_4 and T_3 accumulate

only after the iodination reaction and DIT formation reach a plateau. [62] This study also revealed that the concentration of T_3 levels off after 5-10 minutes, while the T_4 concentration continues to increase. This suggests T_3 is not the precursor of T_4. The MIT/DIT ratio during the time-course of the study supported that DIT is the precursor of T_4.

In most vertebrates, the majority of the circulating thyroid hormones are bound to serum proteins.[63] The major serum thyroid hormone-binding proteins are thyroxine-binding globulin (TBG), thyroxine-binding prealbumin (TBPA), and albumin. The interaction of the hormones with the various binding proteins is noncovalent and reversible. All three binding proteins are synthesized in the liver.[64] They are responsible for the maintenance of a large extrathyroid pool of thyroid hormone, of which only a minute fraction of free hormone is immediately available to tissues. One of the functions of the hormone binding proteins is to safeguard the body from the effect of abrupt fluctuations in hormonal secretion. A second function may be to serve as an additional protection against iodine waste. Body metabolism is dependent on the quantity of thyroid hormones available to peripheral tissues. Since the intracellular hormone is in equilibrium with the free or unbound hormone concentrations in the serum, the free rather than the total hormone concentrations is a more accurate indicator of the hormone-dependent metabolic state.

Thyroxine is predominantly localized to the extracellular compartment. The amount of T_4 secreted by the thyroid is fairly constant; however since the majority of extrathyroidal T_4 is bound to serum binding proteins, fluctuations in T_4 secretion from the thyroid have relatively little influence on the levels of T_4 available to the peripheral tissues. The production of T_3 is primarily from the peripheral conversion of T_4 to T_3.

The distribution of T_4 and T_3 to the tissues in the body is due to a complicated series of relationships between cellular uptake and intracellular binding proteins. In humans 31 percent of T_4 is taken up by the liver and kidney; 44 per cent is found in muscle, brain, and skin; and the remaining 22 per cent is present in plasma. The distribution of T_3 is different, with only 5 per cent present in the liver, 18 per cent in plasma, and 75 per cent in remaining tissues.[65]

The transformation of the iodothyronines require that the hormones enter the cell or be closely bound to the cell membrane. The unbound or "free" thyroid hormones enter the cell. The peripheral deiodination of T_4 results in the formation of T_3 and rT_3. This deiodination is the action of several deiodinases that display both substrate and tissue specificity. In certain tissues such as brain and pituitary, nuclear T_3 is derived in large part from local deiodination of T_4 within the cell, whereas in other tissues such as liver, kidney and heart, the principal source of T_3 is plasma. This

allows certain tissues a degree of autonomy in the regulation of their nuclear T_3.[66]

Mechanisms of Action

Thyroid hormones act on tissues through various mechanisms, including transport of amino acids, and electrolytes from the extracellular environment to the interior of the cell; synthesis or activation of specific enzyme proteins within the cell; and enhancement of intracellular events, including translation and transcription, that lead to changes in cell size, cell number, and cell function.

Although it has been assumed that thyroid hormones enter the cell by passive diffusion, recent studies have suggested that there is an active transport system into target cells of the liver and kidney.[67] To effect their actions, thyroid hormones bind to a nuclear receptor and are capable of acting directly on the genome to initiate transcription or translation. Thyroid hormone receptors are nonhistone chromatin proteins found in nuclear fractions.[68] Nuclear receptors for T_3 have a high affinity but a limited number of receptors and do not require cystolic transport for T_3 to gain access to the cell nucleus.[69]

The dramatic effects of T_3 on O_2 uptake by tissues resulting in an increased "metabolic rate" indicates that thyroid hormones are a prime determinant of metabolic activity which differs qualitatively from tissue to tissue. This

increased metabolic rate is reflected by increased mitochondrial activity in liver, kidney and muscle. Thyroid hormones stimulate the sodium pump which operates through membrane-bound Na^+,K^+-ATPase and glycolytic pathways leading to calorigenesis coupled with oxidative phosphorylation. Other tissue effects include: increased lipolytic activity in adipose tissue; modulation of gonadotropin secretion by the pituitary; and altered concentrations of neurotransmitters in the brain.[70] Tissue responsiveness to thyroid hormones is not static throughout life, but decreases with aging. Thyroid hormones modulate the actions of other hormones through receptor interactions or by altering transcription or translation events initiated by other hormones. Finally thyroid hormones can regulate concentrations of their own receptors and, under certain circumstances, modify, through transamination, their own metabolism.[71,72]

Thyroid Tests

Thyroid function testing in veterinary medicine has improved in the last decade, however, there is still no single laboratory test that will positively prove hypothyroidism. Each of the currently used tests will measure one aspect of thyroid function at a given time. Because of the complexity of the interacting regulatory mechanisms which control thyroid gland secretion, a clinician must understand what a specific test measures, the technical and interpretative difficulties

encountered with the test, the clinical conditions that alter the test, and what further testing may be necessary.

Thyroid testing can be classified according to the information provided at the functional, etiological or anatomical levels. Tests such as thyroidal radioiodide uptake, performed in vivo, directly assess the level of thyroid gland activity and its ability to perform hormone biosynthesis. Tests that measure hormone concentration and its transport in the blood are an indirect assessment of the level of metabolic activity of the thyroid gland. Additionally, the presence of substances, such as thyroid autoantibodies, usually absent in healthy individuals, is useful in establishing the etiology of some thyoid disorders.

Currently, the most common screening test for dogs is the measurement of the circulating iodothyronines, T_4 and T_3. While many laboratory techniques were used in the past, such as column chromatography and the protein-bound iodine test, virtually all tests have been replaced by radioimmunoassays (RIA) or enzyme-linked immunoassays (ELISA).[73] The T_3-uptake tests are still used in humans to measure the degree of plasma protein binding.[74] The T_3-uptake test is considered an insensitive test in the dog as T_3 and T_4 bind less avidly to the canine plasma proteins than does human T_4 and T_3. The normal T_3 uptake in the dog is 40% to 60% of the plasma versus 85% to 95% in human plasma.[75]

The measurement of total T_4 and total T_3 is now done using RIA modified and validated for the dog.[76] The principle behind RIA is the competition of the substance being measured with a known quantity of radiolabeled substance. Specific monoclonal or polyclonal antibodies are used to bind the labeled and unlabeled substances. The antibodies can either be fixed to a solid support, such as a tube lining, or the antibody can be added to the antigen and the antibody-ligand complexes precipitated out of the solution. A standard curve is based on increasing quantities of the antigen being added to the assay and a stoichiometric relationship between the antigen-antibody and antibody-radiolabeled antigen determined. This relationship can be converted to a straight line by mathematical transformations, such as the logit-log plot. The sensitivity of the assay is dependent upon the affinity of the antibody and the specific activity of the radiolabeled antigen.

Many nonthyroidal factors can affect concentrations of serum thyroid hormones. Fasting, systemic illness, drugs and other factors have been shown to lower total T_4 and T_3 concentrations. In humans with either chronic or severe acute non-thyroidal illnesses, inhibitory substances have been found in the circulation which impair the binding affinity of T_4 to serum binding proteins.[77] In conditions of inadequate dietary protein and carbohydrate intake, the low T_3 state appears important in facilitating protein conservation.[76,78] Various

drugs can alter the concentrations of thyroid hormones by affecting any level of the hypothalamic-pituitary-thyroid axis. In addition to altering thyroid hormone synthesis, drugs can affect the transport, metabolism, and excretion of T_4 and its derivatives. Some hormones and drugs may alter the concentration of the binding proteins in the serum affecting the thyroid transport in the circulation. Thyroid binding globulin concentrations are increased by estrogens and decreased by drugs such as glucocorticoids and androgens as well as protein-losing enteropathies, hepatitis, malnutrition, ovariectomy, and nephrotic syndrome.[79-82] In addition to decreasing the serum binding proteins, glucocorticoids also inhibit the conversion of T_4 to T_3 in the peripheral tissues. This results in a decrease in T_3 concentrations with an increase in rT_3 concentrations.[83] Measuring the "free" or unbound T_4 and T_3 is used to measure the concentration of active hormone in the circulation unaffected by the protein-binding. Recent studies in the dog indicate the measurement of unbound T_4 as measured by radioimmunoassay may not be effective in separating hypothyroid dogs from euthyroid dogs with systemic illnesses.[84] Measurement of unbound T_4 by equilibrium dialysis may be a more accurate guide to use to determine concentrations of unbound T_4.[84]

Another test to measure the ability of the thyroid gland to function is the TSH stimulation test. In this test, exogenous TSH is administered after baseline serum thyroid

hormone concentrations are established. Interpretation of the response to TSH depends on the amount of TSH administered and the length of the interval between serum samples. Stimulation with TSH does not cause signs of hyperthyroidism since the elevated concentrations of T_4 are primarily protein bound.[85] The maximum response of T_4 and T_3 concentrations to TSH stimulation has been shown to be between eight and twelve hours post TSH.[86] The dosage and route of administration have not seemed to effect the results significantly, however since bovine TSH is a foreign protein, repeated administration could cause the production of antibodies and an allergic reaction. The normal response to TSH administration is a two to four-fold increase in T_3 and T_4 concentrations. Usually there is no problem differentiating the normal response from primary hypothyroidism, but occasionally when there is a poor response, or the resting values are very low, it may be difficult to differentiate non-thyroidal illness from hypothyroidism.

The thyroid releasing hormone (TRH) stimulation test is used to determine if the pituitary can produce TSH to stimulate the thyroid. Baseline thyroid hormone concentrations are obtained, followed by the administration of TRH. In the dog, the response to this test is so poorly documented, that intrepretation of results is difficult. As a result, this test is rarely performed.

Measurement of canine serum TSH concentrations would be extremely helpful in the diagnosis of thyroid disorders. Unfortunately, at this time no reliable assay is available for commercial use.

A radioactive iodine uptake study can be used to determine the thyroid gland's functional ability to take up iodine against a gradient. Radioactive tracers including, ^{123}I, ^{125}I, or ^{131}I can be used. ^{123}I is preferred due to its lower radiation hazard. Twenty four to seventy two hours following administration of the radioactive tracer, radioactivity counts are taken over the thyroid. This test is very expensive, time-consuming and not generally available. Due to the environmental hazards this test is rarely performed in dogs.

Autoimmune Thyroiditis

Autoimmune lymphocytic thyroiditis is an endocrine disease characterized by either the infiltration of the thyroid gland by lymphocytes or idiopathic follicular atrophy.[87] Spontaneous autoimmune lymphocytic thyroiditis is present in humans (Hashimoto's thyroiditis), dogs, chickens, and BUF rats.[88,89] Clinical signs of hypothyroidism appear as the thyroid parenchyma is destroyed by either invasion of the thyroid epithelium by lymphocytes or by the atrophy of thyroid follicles. Currently it is believed that both of these disorders are autoimmune in origin and involve both cellular and humerol mechanisms, although the exact etiology and

pathogenesis are unknown.[87,90,91] The role of humerol immunity is suggested by the presence of circulating antibodies to various thyroid antigens. In addition, immune complexes of Tg and anti-Tg antibodies have been detected along the follicular basement membrane. These complexes may lead to thyroid damage through complement fixation or by activation of killer cell activity.[90] Antibody-dependent cell mediated cytotoxicity (ADCC) also has been observed in sera from patients with Hashimoto's thyroiditis. Cytotoxic T cells or the cytotoxic effects of lymphokines released by activated T cells responding to thyroid antigens are potential cell mediated immune mechanisms that may also potentially damage the thyroid.[92]

Autoantibodies that react specifically with normal thyroid gland constituents are detected in the serum of patients with autoimmune thyroid disease. The presence of these autoantibodies has been used to investigate the pathogenesis of the disease and also for diagnostic purposes.

Autoantibodies to Tg have been detected in humans, chickens, and dogs with autoimmune thyroiditis. Normally, Tg is present in small quantities in the circulation of up to 90 per cent of euthyroid, healthy adult human beings.[93] Thyroglobulin has also been detected in normal dog sera and appears to be present in higher concentrations than in human beings. The difference in the concentration of serum Tg between human beings and dogs may be due to assay variability

or a different mechanism of release of Tg from the thyroid.[94] Autoantibodies to Tg have been found in about 60 per cent of adults with Hashimoto's thyroiditis.[95] In one study of autoantibodies to human Tg, 1 minor and 2 major epitopes were found on the antigen indicating a polyclonal antibody response with different isoelectric focusing points.[96] Using monoclonal antibodies to human thyroglobulin, it was found that human Tg had 5 to 6 epitopes unique to primate and human Tg in addition to a number of epitopes that cross react with other species.[97] Autoantibodies to Tg appear to be directed against the normal Tg molecule as they do not react with abnormal Tg or Tg that has been heat denatured. This suggests that the autoantibodies to Tg recognize epitopes on 2 or more peptide chain. Complexes of Tg and antibody are deposited in the thyroid gland which may cause thyroid damage and activate killer cells. In human beings, Tg autoantibodies are primarily of the IgG class of immunoglobulin, of the subclass 1.[98] However a small percentag of the autoantibodies are IgG subclass 4, which fixes complement.[99] In addition to IgG, anti-Tg IgA has been detected in 40 to 50 per cent of human patients with Hashimoto's thyroiditis.[100] This is important as IgA has been shown to be involved in complement induced tissue damage in other organs. Generally, Tg autoantibodies do not fix complement nor are they cytotoxic. The presence of Tg autoantigens on the thyroid cell surface membranes makes them susceptible for potential damage by autoantibodies. The

demonstration that partially glycosylated asialoagalacto Tg, which bears the parent molecule's autoantigenic determinants, is expressed on the thyroid follicular cell surface suggest a potential pathogenetic route for this antigen in autoimmune thyroid disease.[101]

Autoantibodies to T_4 and/or T_3 have been reported in all species reported to develop autoimmune thyroiditis.[88,89] As discussed previously, the majority of circulating T_4 and T_3 is bound to serum binding proteins such as thyroxine-binding globulin, prealbumin and albumin. Thyroid hormones can be considered haptens and thus are unable to induce antibody formation on their own. Much research has been done to study the underlying immunologic mechanism of the production of thyroid hormone autoantibodies. Thyroglobulin contains large quantities of T_4 and T_3 that could provide antigenic sites for the formation of autoantibodies to T_4 and T_3. Thyroid autoantibodies have been found to cross-react with the thyroxyl-containing sequences of Tg and thus may be a subset of the Tg autoantibodies.[102] However, because thyroid hormone autoantibodies have been detected in sera samples with no autoantibodies to Tg under certain pathologic conditions other serum proteins may also be involved with the formation of thyroid hormone autoantibodies.[103] Clinically, thyroid hormone autoantibodies in the patient's serum interfere with RIA of thyroid hormones. This interference results in confusing information about the patients' thyroid status.

Depending on the type of RIA used, the serum thyroid hormone concentrations can appear to be very high or very low. With a single-antibody RIA technique using either polyethylene glycol or absorption or using free thyroid hormones with charcoal dextran, the thyroid hormone autoantibodies behave in the same manner as the radiolabeled hormones added to the assay. Thus, as levels of bound ^{125}I-T_4 or ^{125}I-T_3 increase, the value of T_4 or T_3 determined from the standard curve shows a lower than actual value. The opposite result is seen with double-antibody assays, in which the binding of labeled thyroid hormone by the autoantibodies results in less radioactivity being bound by the assay antibodies which are added first. The second antibody recognizes only the assay antibodies, not the autoantibodies against thyroid hormones resulting in less tracer hormone being precipitated causing falsely higher results.

The degree of interference of thyroid autoantibodies on the RIA is to some extent a reflection on their binding potential, including their affinity and concentration. Copping et al.(1989) used reverse-flow electrophoresis to demonstrate that autoantibodies are capable of transporting thyroid hormones in vivo in the presence of the other serum binding proteins.[104] We demonstrated that the autoantibodies continue to bind thyroid hormones in the presence of 8-anilino-1-naphthalene-sulfonic acid (ANS)in dogs, while binding to the other serum binding proteins was blocked.[105]

This confirmed that the thyroid autoantibodies had both the affinity and the concentration required to be present in the equilibrium between the thyroid hormones and the binding proteins.

The failure of the thyroid hormone autoantibodies to induce hypothyroidism by their binding to thyroid hormones in circulation is not surprising as they function along the lines of the other serum binding proteins, such as TBG, prealbumin and albumin. As long as the patient has sufficient thyroid reserve, enhanced plasma binding of T_4 (due to thyroid autoantibodies or serum binding proteins) produces a transient decrease in unbound T_4 which serves as a stimulus for enhanced T_4 secretion via negative feedback resulting in a new steady state with an increase in total T_4 but no change in the unbound T_4. These observations have been confirmed in animals with experimentally induced thyroid autoantibodies; the unbound T_4 serum concentrations was reported normal while total T_4 serum concentrations were markedly elevated.[106]

The microsomal antigen has been shown to be the membrane bound, glycosylated enzyme, TPO. These autoantibodies have been shown to be highly correlated to thyroid pathology in human beings, with approximately 95 per cent of adults with Hashimoto's thyroiditis having anti-TPO autoantibodies.[107] It has been shown that fusion of the exocytotic vesicles carrying the newly synthesized Tg with the apical plasma membrane leads to cell surface expression of the TPO. The exposed TPO

results in complement-mediated cytotoxicity in sera from patients with autoimmune thyroid disease.[108] Using primary cultures of human thyroid cells, it was shown that the expression of TPO antigen in thyroid cells is dependent on TSH stimulation, through pathways which involve cAMP production and synthesis.[109]

It has also been reported that autoantibodies to TPO are capable of inhibiting the catalytic activity of TPO.[110] Okamoto et al. (1989) found that the mean serum free T_4 concentration was significantly lower in patients with TPO autoantibodies suggesting that these autoantibodies inhibit thyroid function, but no simple relationship between TPO autoantibodies and thyroid function was demonstrated.[111] It is difficult to assume that TPO autoantibodies inhibit the activity of the enzyme, because of its inaccessibility to the cytoplasm of thyroid follicular cells.

A study by Kohno et al.(1986) found that IgG fractions purified by ion exchange chromatography or protein A-Sepharose column from human patients with chronic thyroiditis inhibited the activity of TPO by both guaiacol assay method and iodide assay method.[112] This same study suggests that the antigenic determinants on the TPO molecules are heterogeneous with two binding sites, one for the tyrosyl residue in Tg and one for the aromatic donor. Recognition of the inhibition of TPO activity by either the guaiacol assay or the iodide assay

might be caused by autoantibodies which recognize either one of the sites, both sites or none.[113]

The effector mechanisms for tissue damage in Hashimoto's thyroiditis in human beings is fairly well defined with the interplay of several separate factors. The important cytotoxic effects of microsomal autoantibodies are recognized.[114] Antibodies can also be demonstrated in immune complexes deposited on the follicular basement membranes and these can fix complement to mediate tissue damage. ADCC may be important in Hashimoto's thyroiditis as supported by the increase in killer cells which produce this form of cytotoxicity.[115] Although the evidence points toward ADCC, the role of antibody-dependent complement-mediated cytotoxicity and direct T cell killing as relevant pathogenetic mechanisms in the human disease is more complex. What initiatesthe process off appears to be related to the role of the antigen-presenting cell.[116] It has been demonstrated that patients with Hashimoto's thyroiditis have aberrant expression of class II HLA antigens on thyroid cells, probably in response to stimulation by lymphocyte-secreted interferon-γ.[116-119] It has been further suggested that the thyroid follicular cells may have the capacity to present their own self-autoantigens in conjunction with the class II antigen to the immune system.[120]

Considering that most patients with Hashimoto's thyroiditis express HLA-DR antigens in the thyroid epithelial cells, and that TSH enhances the interferon-γ-induced HLA-DR

expression in cultured human thyrocytes, TSH may pay a role in the expression and/or maintenance of thyroid immune responses in Hashimoto's thyroiditis by developing TPO antigens.[121,122]

Although the pathogenesis of autoimmune thyroiditis in human beings has been extensively studied, the cause cannot be attributed to abnormal T cell function. Lymphocytes of patients with lymphocytic thyroiditis generally do not have a positive blastogenic response to Tg, although in one study 71 per cent of human patients with lymphocytic thyroiditis and 9 per cent of the normal control patients had a positive response to Tg.[123] Another test for T cell function uses the production of the lymphokine, migration inhibition factor (MIF) released when T lymphocytes encounter an antigen to which they are sensitized. The results of this test suggest an antigen-specific defect in suppressor T cell function.[124] When T cells are cloned from the thyroid tissue of patients with Hashimoto's thyroiditis, a large number are reported to have the $T4^{+}T8^{+}$ phenotype of cytotoxic/suppressor cells and are cytolytic.[125]

Investigations of B lymphocyte cell function in human patients with Hashimoto's thyroiditis found no generalized B cell hyper-reactivity to exogenous antigen. When cultured B cells were stimulated with pokeweed mitogen (PWM) no suppressor effect by normal T cells on autoantibody production was observed and normal T cells could provide efficient helper function.[126] In contrast, when normal B cells were combined

with T cells from patients with Hashimoto's thyroiditis no Tg autoantibodies were made.[127,128] With the use of lymphocytes freshly collected from the thyroid glands of patients with Hashimoto's thyroiditis, spontaneous Tg autoantibody formation synthesis occurred without PWM stimulation; thus, the target organ is an important source of autoantibodies in autoimmune thyroid disease.[129]

Hypothyroidism is an important endocrine disease in dogs and is often associated with dermatologic abnormalities such as alopecia, seborrhea and pyoderma. Other clinical signs seen in hypothyroid dogs include obesity, lethargy, hypothermia, bradycardia and neuromuscular disorders.

Autoantibodies to thyroid antigens have been detected in canine serum suggesting the presence of immune mediated thyroid disease. Previous studies have documented autoimmune thyroiditis as the most common cause of canine hypothyroidism.[130,131] While both the idiopathic follicular atrophy and the lymphocytic forms have been documented in the dog, lymphocytic thyroiditis is more common.[130]

Idiopathic thyroiditis is characterized by loss of the thyroid parenchyma and replacement by adipose and connective tissue. It lacks the inflammatory infiltrate seen with lymphocytic thyroiditis. Degeneration of follicular cells may be seen.[132] Lymphocytic thyroiditis is characterized by a focal to diffuse infiltration of the thyroid gland by lymphocytes, plasma cells, and macrophages. Thyroid follicle

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destruction and lymphocytic nodule formation may be present as the disease progresses. In advanced cases fibrosis with minimal inflammatory infiltrate remaining may be seen.[133]

A study of the progression of the disease by Conaway et al (1985), indicated three stages. Early changes were noninflammatory and characterized by slight follicular degeneration causing collapse of follicular lumens. The second phase was inflammatory with infiltration of lymphocytes, plasma cells and macrophages. Ultrastructural examination at this time noted focal thickening of the basal lamina, suggesting immune complex deposits. The final stage is fibrous connective tissue replacement of the normal thyroid parenchyma. One of the dogs in the study had end-stage thyroiditis with histologic findings similar to idiopathic thyroid atrophy.[134]

In human beings, lymphocytic thyroiditis is more prevalent in women; however this does not appear to be true in the dog with males and females equally affected.[135-137] A familial tendency has demonstrated in Great Danes, beagles and Borzois.[136-138] An increased prevalence of thyroid autoantibodies in some breeds including Doberman Pinchers, Old English Sheepdogs, and Irish Setters suggests that thyroiditis is inherited in these breeds also.[138,139] A definite pattern of heritability has not been established in the dog as of this time, however it appears to be polygenic as in human beings.[140] In a study with Borzoi dogs lymphocytic thyroiditis appeared

to be an autosomal recessive trait.[135] Further studies are needed to confirm the mode of inheritance.

In a colony of laboratory Beagles, a 16.2% incidence of thyroiditis was detected in young adults of both sexes.[136] The presence of thyroid autoantibodies has also been detected in dogs with lymphocytic thyroiditis.[105,141-144] Autoantibodies to canine Tg have been detected by tanned cell hemagglutination, chromic chloride passive hemagglutination and ELISA techniques. The incidence of Tg autoantibodies varied with the type of assay and the criteria used to select the population of dogs to be tested. However, the incidence of Tg autoantibodies detected in dogs with hypothyroidism is very similar to the incidence of autoantibodies in humans with Hashimoto's thyroiditis.[105,141,142,144] In addition to Tg autoantibodies, autoantibodies to the thyroid hormones, T_4 and T_3 have been detected in dogs with lymphocytic thyroiditis.[105,145-147] Complement fixation and ELISA techniques have been used previously to measure microsomal autoantibodies in the dog,[142,143] These studies found a low incidence of microsomal autoantibodies in dogs, however, since these studies were conducted, the techniques to isolate and purify thyroid microsomes have been greatly improved.

Haines et al (1984) investigated the presence of antithyroglobulin (anti-Tg) autoantibodies and thyroid microsomal autoantibodies in 34 dogs with low basal serum T_4 concentrations.[142] Autoantibodies to thyroid microsomal

autoantibodies were detected by ELISA in 29% of those dogs. In contrast, autoantibodies to Tg were found in 59% of the dogs. Autoantibodies to Tg have also been detected in the sera of dogs with non-thyroidal endocrine disorders. In one study, 43% of 65 dogs with nonthyroidal endocrine disease had autoantibodies to Tg as detected by ELISA, however, Gosselin et al (1980) did not detect autoantibodies to Tg using chromic chloride hemagglutination in sera from a similar population of dogs.[141] Human patients with immune-mediated endocrine disorders which resulted in diabetes mellitus or hypoadrenocorticism are predisposed to the development of thyroid autoantibodies.[148] Relatives of dogs with lymphocytic thyroiditis commonly are reported to have antibodies to Tg, and the same is true in people.[138,148]

In a study in our lab of canine sera collected from dogs suspected of having autoimmune thyroiditis resulting in clinical hypothyroidism, we detected autoantibodies to T_4 and T_3 in 26.1% and 32.8% respectively.[105] Of the 119 serum samples used in the study, 2.5% had autoantibodies to only T_4, 0.8% had autoantibodies to only T_3, and 3.4% had autoantibodies to T_4 and T_3. This suggests that autoantibodies to the thyroid hormones may bind to another protein occasionally in the dog to induce the production of these autoantibodies. In an earlier study of dog serum submitted for the measurement of thyroid hormone concentrations, only 0.2% of the samples were found to have autoantibodies to T_4 and/or T_3, however

identification of these autoantibodies was based on a marked alteration in the results of RIA of thyroid hormones, not the direct demonstration of autoantibodies.[147] In studies of thyroid hormone autoantibodies in human beings, the prevalence of autoantibodies to T_4 and T_3 was found to be dependent on method of detection, ranging from 40% (6 of 15) detected by electrophoresis techniques to 2.6% (1 of 38) from a modified RIA technique.[149,150]

Mizejewski et al (1971), used complement fixation to measure microsomal autoantibodies in a colony of beagle dogs.[143] Using that assay, 7 of 11 dogs with thyroid pathology had significantly high titers to complement fixing antibodies. Fluorescent antibody staining was also performed on thyroid biopsies from these dogs. A staining pattern characterized as "cracked mirror" appearance as described by Balfour et al (1961), suggesting the presence of both Tg and microsomal autoantibodies was seen in the thyroid glands in the same 7 dogs.[151] In other studies, immunofluorescence methods were found to be technically unreliable and time consuming.[130] It appears that in the dog, autoantibodies to Tg are more prevalent than the microsomal antigen. This is in direct conflict with results found in humans, but is similar to chickens with spontaneous autoimmune thyroiditis and mice.[149,150] In a study of Tg autoantibodies using both an ELISA and Western blot assay, there was a significant correlation, however, some sera were positive on the Western blot and

negative on the ELISA and some sera were positive with the ELISA and negative on Western blot assay. This suggests a heterogeneous population of antibodies.

The recognition that the major component of the autoimmune response is generated within the thyroid and that the thyroid has the capacity to invoke these responses now makes the thyroid not only the target of the autoimmune response but also the source.[128] What initiates these events is unknown, but environmental influences are suspected. Iodine ingestion is increasingly implicated in the possible generation of the autoimmune response.[12]

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Prevalence of autoantibodies to thyroglobulin, thyroxine, or triiodothyronine and relationship of autoantibodies and serum concentrations of iodothyronines in dogs

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SUMMARY

Assays were developed to detect and measure autoantibodies (AA) to thyroglobulin (Tg) and to the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3). An ELISA to detect AA to Tg was developed, using purified canine Tg as the antigen and goat anti-canine IgG conjugated with alkaline phosphatase as the second antibody. A highly charged agarose electrophoresis assay was used for determination of AA to T_4 and T_3. Sera from dogs (n = 119) with clinical signs consistent with hypothyroidism were tested for AA to Tg, T_4, and T_3. Autoantibodies to at least 1 of the 3 thyroid antigens were detected in 58 of the 119 (48.7%) sera tested. Autoantibodies to Tg were detected more frequently in samples with low serum concentrations of thyroid hormones than in samples with normal concentrations. The presence of AA to T_4, T_3, or both was not significantly associated with low thyroid hormone concentrations, but this lack of association may have been attributable to binding of AA in the measurement of thyroid hormones by radioimmunoassay.

Autoimmune thyroiditis in dogs is an endocrine disease characterized by lymphocytic infiltration of the thyroid gland.[1] Although the pathogenesis is unknown, cellular and humoral immune mechanisms appear to be involved.[2,3] Cell-mediated immune mechanisms found in human beings with autoimmune lymphocytic thyroiditis include the actions of cytotoxic T cells or the cytotoxic effects of lymphokines released by activated T cells responding to thyroid antigens.[4-6] The role of humoral immunity is suggested by the detection of circulating antibodies to various thyroid antigens. Additionally, immune complexes of thyroglobulin and antithyroglobulin antibodies have been found along the thyroid follicular basement membranes of human beings with autoimmune lymphocytic thyroiditis.[1,6,7] In dogs and human beings, clinical signs of hypothyroidism develop as the thyroid gland is destroyed, either by lymphocytic invasion of the thyroid follicular epithelium or by atrophy of thyroid follicles.[8]

Hypothyroidism in dogs is routinely diagnosed by measuring serum concentrations of thyroid hormones, with or without prior administration of thyroid-stimulating hormone (TSH).[9,10] However, concentrations of thyroid hormones frequently are not affected until the later stages of thyroid disease, and, therefore, are of limited value in the early diagnosis of lymphocytic thyroiditis. Additionally, other physiologic factors, such as systemic disease and drug treatment, may lower hormone concentrations in dogs without primary thyroid disease.

Autoantibodies (AA) directed against the following thyroid antigens have been found to be important in the diagnosis of lymphocytic thyroiditis in human beings: thyroglobulin (Tg); a microsomal antigen identified as thyroid peroxidase; thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3); and a colloid antigen.[11-13] Studies have identified AA to Tg, T_4, T_3, microsomal antigen, and colloid antigen in dogs; however, the association between various AA and clinical disease varied among studies.[14-17] Improving the assays used in the detection of these AA may aid in early diagnosis of autoimmune thyroid disease in dogs. The objectives of the study reported here were to develop sensitive and specific assays to detect AA to Tg, T_4, and T_3 in dogs, to assess the prevalence of these AA in dogs having clinical signs consistent with hypothyroidism, and to determine the relationship between AA and serum concentrations of thyroid hormones.

Materials and Methods

Samples—Negative reference sera usd in the ELISA for detection of AA to Tg were obtained from 13 dogs with normal serum concentrations of thyroid hormones, as determined by radioimmunoassay (RIA); dogs were euthanatized, and microscopic evidence of lymphocytic thyroiditis was not found on histologic examination of thyroid glands. Sera also were obtained from 2 dogs given repeated injections of purified canine Tg in adjuvant*; these samples were designated as positive reference sera in the ELISA for Tg AA. In the agarose gel electrophoresis assay for detecting T_4 and T_3 AA, serum from a dog with high T_4 concentrations and low T_3 concentrations on RIA, consistent with AA to T_3 and T_4, was designated as positive reference serum. Autoantibodies to T_4 and T_3 interfere with measurement of hormone concentrations by RIA by binding to the radioactive ligand.[19-22]

Sera designated as test samples (n = 119) in this study

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were originally submitted for determination of thyroid hormone concentrations and were obtained from dogs with reported clinical signs of alopecia, lethargy, and obesity. Dogs with pruritus, or dogs that were given exogenous thyroid hormones or glucocorticoids, were excluded.

The ELISA methodology—Purified canine Tg,[b] diluted in phosphate-buffered saline solution (PBSS, pH 7.4) was used as the antigen in the ELISA for detection of AA to Tg. Fifty microliters of this solution (10 μg/ml) was added to polystyrene microtitration trays[c] and incubated for 16 hours at 4 C. After the antigen was fixed with 50 μl of 0.5% glutaraldehyde, the wells were emptied and washed 3 times with PBSS containing 4% bovine serum albumin (BSA) and 0.05% Tween 20 (BSA-Tween in PBSS). A titration curve of serum dilutions from 1:20 to 1:1,280 was constructed from triplicate testing of the positive and negative reference sera. Test sera and positive and negative reference sera were diluted 1:50, 1:100, and 1:200 in 0.5% BSA in PBSS, and 50 μl of each were added to wells and incubated at 25 C for 2 hours. All samples were run in duplicate. Fifty microliters of 0.1*M* diethanolamine solution (pH 9.8) also was added to each well, and incubated at 25 C for 30 minutes. The wells were emptied and washed 3 times with BSA-Tween in PBSS. To each well, 50 μl of goat anti-canine IgG conjugated with alkaline phosphatase,[d] diluted 1:100 in 0.5% BSA in PBSS was added, and wells were incubated at 37 C for 1 hour. The wells were washed twice with BSA-Tween in PBSS and then washed with double-distilled water. To each well, 50 μl of the enzyme substrate, p-nitrophenyl phosphate (1 mg/ml) in 1.0*M* diethanolamine buffer solution (pH 9.0), was added, and wells were incubated for 20 minutes at 37 C. The enzyme reaction was stopped by the addition of 50 μl of 1.0*N* NaOH, and the absorbance was read on a spectrophotometer[e] at 405 nm.

For 16 hours at 4 C, 500 μl of positive and negative reference sera were incubated with 50 μl of Tg, T_4, or T_3 in increasing concentrations (0 to 2.36 mg/ml, 0.25 to 20 ng/ml, and 5 to 100 ng/ml, respectively) to determine specificity of the ELISA.

Agarose gel electrophoresis—Autoantibodies to T_4 and T_3 were assayed, using a highly charged agarose[f] gel slab electrophoresis technique modified from Brown et al.[23] Sera (50 μl) were incubated for 16 hours with 150 μCi ^{125}I-labeled T_4 and T_3 (750 to 1,250 μCi/μg)[g] and were electrophoresed for 3 hours. Autoradiographs of the gels were made to identify appropriate portions of the gels to be excised, and the radioactivity was counted by a gamma counter.[h] To confirm IgG migration toward the cathode, 10 μg of purified canine IgG[i] also was electrophoresed.

To determine the effect of endogenous T_4 and T_3 on the binding of AA and antigens, thyroid hormones were removed from serum by use of a modified technique of Sakata et al.[19] To remove endogenous T_4 and T_3, 100 μl of positive and negative reference sera each were incubated for 16 hours with 100 μl of 0.05*M* glycine-HCl buffer solution with 2.5% dextran and 0.25% charcoal (pH 2.2).

The effect of thyroxine-binding globulin (TBG) on the binding of AA to T_4 and T_3 and ^{125}I-radiolabeled T_4 or T_3 was assessed by adding 8-anilino-1-naphthalene-sulfonic acid (ANS, 90 mg/ml) to the positive and negative reference sera. Bound iodothyronines are displaced from serum-binding proteins including TBG, thus allowing radiolabeled hormones to bind to the AA.

Measurement of thyroxine and triiodothyronine—Serum concentrations of total T_4 were measured by RIA with a solid-phase assay modified for use in dogs. Serum T_3 concentrations were determined by a charcoal-separation RIA technique validated for use in dogs.[18]

Statistics—Chi-square analysis was used to assess significant association between serum iodothyronine concentrations and detection of AA. A P-value < 0.05 indicated statistical significance.

Results

Evaluation of ELISA technique—A dilution of 1:100 provided acceptable separation of positive and negative reference sera (Fig 1). Mean absorbance of positive reference sera at a 1:100 dilution was 0.299 (SD, 0.096), whereas the negative sera had a mean absorbance of 0.011 (SD, 0.009). Absorbance values greater than 0.030, or more than 2 SD greater than the mean absorbance for the negative reference sera at a dilution of 1:100, were considered positive for AA to Tg. The intra-assay coefficient of variation (CV) for a pooled sample of negative reference sera was 12.2%, and the interassay CV for the same sample was 8.6% (n = 4 assays).

Specificity of the ELISA for AA to Tg was confirmed by decrease in absorbance as the positive reference serum was incubated with increasing amounts of Tg (Fig 2). Absorbance was not changed when the positive reference serum was incubated with increasing concentrations of T_3 and T_4.

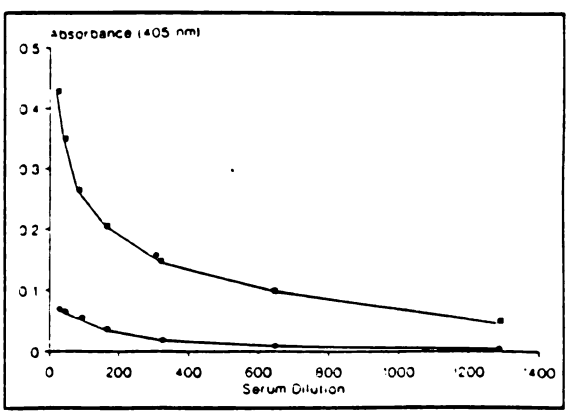

Figure 1—The effect of dilution (1:20 to 1:1,280) on absorbance in positive (■—■) and negative (●—●) reference serum samples in the ELISA for thyroglobulin (Tg). Each point represents a mean value of 8 assays. Serum dilutions of 1:100 (serum dilution = 100 on plot) were adequate to differentiate sera with autoantibodies (AA) from negative sera.

[b] Provided by Dr. Tom Brown, Wayne State University, Detroit, Mich.
[c] Corning Glass Works Inc, Corning, NY.
[d] Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md.
[e] Model EL 307, Bio-tek Laboratories, Burlington, Vt.
[f] Agarose High-M., Bio-Rad Laboratories, Richmond, Calif.
[g] NEN Research Products, Dupont Co, Boston, Mass.
[h] Model 1190, Tm Analytic, Elk Grove Village, Ill.
[i] Cappel Laboratories, Malvern, Pa.

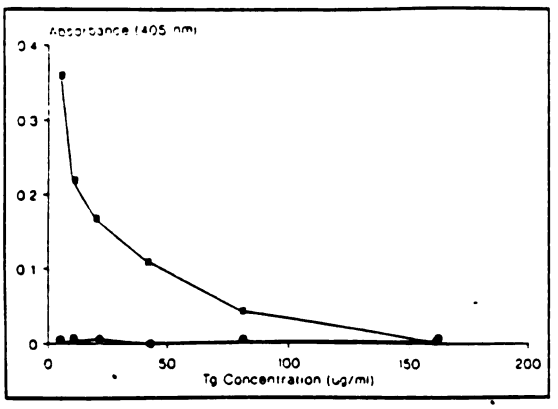

Figure 2—Effect of increasing concentrations of Tg on absorbance in positive (■—■) and negative (●—●) reference serum samples in the ELISA for AA to Tg. Each point is the mean of triplicate determinations. Specificity of the ELISA is demonstrated by the decrease in absorbance with the addition of increasing amounts of Tg.

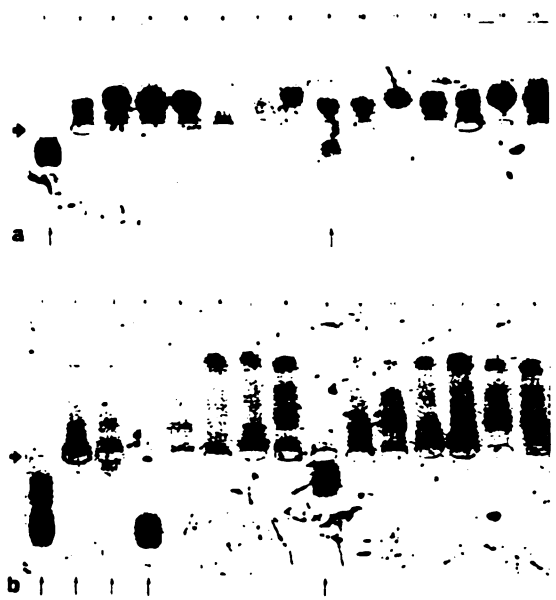

Figure 3—Autoradiographs of agarose gels from sera incubated with ^{125}I-labeled thyroxine (T_4; a) and ^{125}I-labeled triiodothyronine (T_3; b). Origin is large arrow; the small arrows indicate sera with evidence of AA. In Figure 3a, lanes 1 and 9 have 79.4 and 30.1%, respectively, of ^{125}I-labeled T_4 binding in the immunoglobulin region. In Figure 3b, values for ^{125}I-labeled T_3 binding ranged from 28.9 to 86.1% for lanes 1 to 4 and 9.

Evaluation of agarose gel electrophoresis technique—Migration of IgG toward the cathode was confirmed by electrophoresis of purified canine IgG and canine sera in the highly charged agarose. Purified canine IgG migrated only toward the cathode, whereas positive and negative reference sera migrated toward the anode and the cathode, depending on the polarity of serum proteins.

Negative reference serum samples had a mean of 1.67% (SD, 1.28) of radiolabeled T_4 binding in the immunoglobulin region, and 0.94% (SD, 1.11) of radiolabeled T_3 in the immunoglobulin region. The mean value of radiolabeled T_4 in the immunoglobulin region for the positive reference sera was 74.335% (SD, 1.63); for T_3, the mean value was 19.22% (SD, 2.47). The intra-assay CV for negative reference sera was 8.52% for T_4 and 6.32% for T_3. Values > 4%, which was approximately 2 SD greater than the mean percentage for negative reference sera, were considered positive for AA to T_4 and T_3 (Fig 3).

Endogenous T_4 and T_3 had no apparent effect on detection of AA to T_4 or T_3, because removal of the hormones with charcoal dextran solution from reference sera had no effect on the percentages of radiolabeled hormones migrating to the immunoglobulin region. Addition of ANS to these sera resulted in displacement of radiolabeled thyroid hormones from TBG to albumin; however, effects on the binding of hormones in the immunoglobulin region were not found.

Prevalence of AA to Tg, T4, and T3—Of the 119 test samples, 58 (48.7%) had AA to at least 1 of the 3 antigens (Table 1). Autoantibodies to Tg, alone or with AA to T_4, T_3, or both, were found in 50 samples (42%). Autoantibodies to T_4 or T_3 were found in 31 (26.1%) and 39 (32.8%) of the samples, respectively. Of the 58 samples that had positive results, 19 (32.7%) had AA to all 3 antigens. Sera with AA to Tg and T_3 were found most frequently (25.9% of samples with positive results) whereas sera with AA to only T_3 were found least frequently (1.7%).

Association of serum hormone concentrations and AA to Tg, T_4, and T_3—Concentrations of T_4 in the sera tested ranged from 4.7 to 150 ng/ml (normal, 17 to 40 ng/ml), whereas T_3 values were 0 to 1.65 ng/ml (normal, 0.75 to 2 ng/ml). Serum concentrations of T_4 or T_3 were within the normal range in 67 (56.3%) and 40 (33.6%) of the test sera, respectively. Six of the 119 samples had abnormally high or low thyroid hormone concentrations, consistent with interference with the RIA from AA to thyroid hormones.

Autoantibodies to Tg, alone or with AA to T_4, T_3, or both, were detected more frequently ($P < 0.05$) in samples with low serum concentrations of one or both hormones (Fig 4; Table 1). When AA to only thyroid hormones were

Table 1—Distribution of AA to thyroid antigens in serum samples from 119 dogs with clinical signs of hypothyroidism. Fifty-eight (48.7%) of the samples tested had AA to Tg, T_4, or T_3.

Antigen	No. of positive results	Positive results (% of all samples)	Positive results (% of samples having AA)
Tg	11	9.2*	19.0
Tg and T_4	5	4.2*	8.6
Tg and T_3	15	12.6*	25.9
T_4	3	2.5	5.2
T_3	1	0.8	1.7
T_4 and T_3	4	3.4	6.9
All	19	15.9*	32.8

* Significantly ($P < 0.05$) associated with low serum T_4 or T_3 concentrations.
AA = autoantibodies; Tg = thyroglobulin; T_4 = thyroxine; T_3 = triiodothyronine.

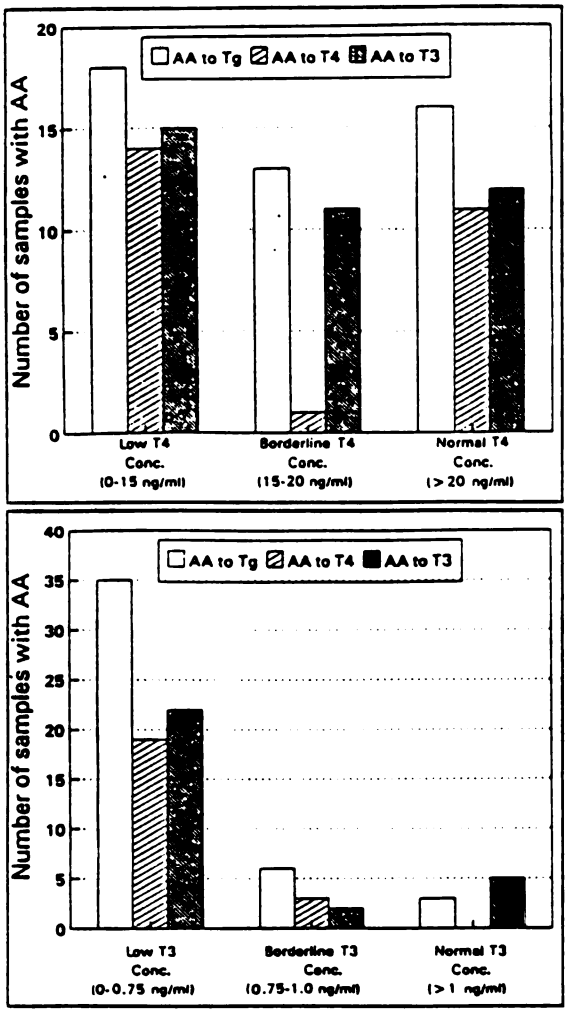

Figure 4—Distribution of AA to Tg, T_4, and T_3 in samples with low to normal serum T_4 (top) and T_3 (bottom) concentrations. Autoantibodies to Tg, with or without AA to T_4, T_3, or both, were more frequently detected ($P < 0.05$) in samples with low serum T_4 or T_3 concentrations.

evident, significant association was not found between serum iodothyronine concentrations and detection of AA.

Discussion

Autoantibodies to at least 1 of 3 thyroid antigens were detected in 48.7% of the sera tested in this study. Autoantibodies to Tg were detected in 42% of 119 samples; similar percentages were found in previous studies of AA to Tg in dogs.[14,17,21]

Autoantibodies to T_4, T_3, or both, with or without AA to Tg, were found in 39.5% of the samples. The agarose gel electrophoresis technique provided a sensitive and reproducible method for measuring AA to T_3 and T_4. There was a wide range of binding in the immunoglobulin region in samples that had AA, whereas in samples with negative results, the amount of radioactive binding was low. The use of 4% binding of the radiolabeled hormone as the cut-off point may be conservative, especially for AA to T_3, because the binding of T_3 for negative reference sera was < 1%. In an earlier study, AA to T_3 were found in only 0.2% of canine sera, but AA detection was on the basis of marked alterations in the results of RIA of thyroid hormones, not on the direct demonstration of AA to T_3.[20] In several studies of human beings with autoimmune lymphocytic thyroiditis, the prevalence of AA to T_4 and T_3 was found to be dependent on method of detection, ranging from 40% (6 of 15) detected by electrophoresis techniques to 2.6% (1 of 38) from a modified RIA technique.[24,25]

Because T_4 and T_3 are haptens, and alone are unable to induce antibody production, Tg has been suggested as the autoantigen for formation of AA to T_4 and T_3.[26] Thyroglobulin contains large quantities of T_4 and T_3 that could provide antigenic sites for development of AA of T_4 and T_3. In this study, 7 samples had AA to T_4, T_3, or both, but not to Tg, suggesting that Tg may not be the only immunogen for the production of AA to T_4 and T_3. Similarly, human beings with AA to T_4, T_3, or both, but not to Tg, have been identified, suggesting that a serum protein, other than Tg, might bind with the hormones in certain pathologic conditions and result in production of AA that bind to thyroid hormones.[27,28]

The electrophoretic heterogeneity of AA to T_3 and T_4 may be attributable to variation in the population of AA. The variation in percentage and migration pattern would suggest that the antibodies are polyclonal, and not monoclonal, which may be important in the study of the pathogenesis of lymphocytic thyroiditis.[23] Production of polyclonal AA is suggestive of lymphocytes responding to multiple autoantigens within the thyroid, whereas production of monoclonal AA is suggestive of either a B-cell malignancy or an easily induced immune response.

Approximately half (56.3% for T_4, 33.3% for T_3) of the serum samples tested in this study had normal thyroid hormone concentrations by RIA. A significant association was found between serum thyroid hormone concentrations and the frequency and type of AA. Detection of AA to Tg, alone or with AA to T_4, T_3, or both, was significantly associated with low thyroid hormone concentrations. Detection of AA to one or both thyroid hormones without AA to Tg was not associated with thyroid hormone concentrations; however, discrepancy between measured serum thyroid hormone concentrations and actual concentrations must be considered when the sample contains AA to T_4 and T_3. In some serum samples with measured T_4 concentrations in the normal range, actual values may have been low, because of AA and RIA antibodies competitively binding to T_4 in serum.

Previous studies have shown correlation between AA to thyroid antigens and lymphocytic thyroiditis in dogs.[14,16,17,29] We suggest that assays that detect these AA, combined with measurement of thyroid hormone concentrations, may be useful in the diagnosis of thyroid disease in dogs.

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GOLDEN RETRIEVER STUDY

SUMMARY

Hypothyroidism is a common endocrine disorder in dogs. Lymphocytic thyroiditis is the most common cause of canine hypothyroidism. The presence of autoantibodies to the thyroid antigens; Tg, T_4, and T_3 have been shown to be associated with lymphocytic thyroiditis in dogs.[1] While the presence of thyroid autoantibodies have been shown to associated with lymphocytic thyroiditis, nothing is known as to how early these autoantibodies occur in dogs and how they relate to the development of thyroid disease.[2] A familial tendency has been determined in Great Danes, beagles, and Borzois.[3,4] Conway, et al (1985) found evidence of a autosomal recessive genetic pattern in Borzois.[5]

The objective of this study was to study a normal population of dogs to determine the incidence of thyroid autoantibodies according to age and clinical signs. Because Golden Retrievers are a popular breed with a fairly high incidence of thyroid disease, we selected this breed for our study.[6] The first population of dogs from which serum was collected was from clinically normal show dogs. A second population of related dogs traced from a Golden Retriever in the first population which had autoantibodies to Tg, T_4, and T_3 was investigated to determine if the presence of thyroid autoantibodies might have a familial basis.

MATERIALS AND METHODS

Populations and serum samples Negative reference serum used in the ELISA and the agarose gel electrophoresis was obtained from 13 dogs with normal serum thyroid hormone concentrations, no evidence of autoantibodies to Tg, T_4, or T_3, and upon euthanasia had no histologic evidence of thyroid disease.

Test sera (n=135) were obtained from Golden Retrievers at the Fort Detroit Golden Retriever Specialty Dog Show. All owners of the dogs answered questionnaires about possible clinical signs of hypothyroidism and any prior history of thyroid disease or diagnostic work. Any dogs which had been administered thyroid medication were eliminated from the study as were dogs on glucocorticoids. Sera for the second population (n=42) of dogs was from relatives of one dog in the original population that contained autoantibodies to Tg, T_4, and T_3. All dogs were included in this study, only the primary dog was on thyroid replacement therapy.

Autoantibody and Thyroid Hormone Assays

The assay useds on all sera obtained from the Golden Retrievers was previously described in chapter 3.

Statistics Chi-square analysis was used to assess relationships between the ages of the dogs and the presence of autoantibodies. Fishers' exact test was used to evaluate significant association between the sexes of the dogs and the

presence of autoantibodies. A P-value <0.05 indicated statistical significance.

RESULTS

The results of the first population of Golden Retrievers from the dog show are shown in Table 2. An overall percentage of 16.3% of the dogs had evidence of autoantibodies to Tg, T_4, or T_3. Autoantibodies to Tg alone were present most frequently, making up 40.0% of the positive samples. Autoantibodies to only T_3 were present in 27.3% of the positive samples with autoantibodies to T_4 being seen with the least frequency. Twenty-two (22.7%) percent of the sera had autoantibodies to more than 1 antigen.

Of the dogs in the first population, only 2 dogs had clinical signs suggestive of hypothyroidism as defined by mild lethargy and slight obesity. The ages of the dogs tested ranged from 5.5 months to 11 years. The ages of dogs with thyroid autoantibodies ranged from 7.5 months to 8.5 years with a mean of 2.6 ± 2.3 years. Autoantibodies were found to be present most frequently ($P<0.05$) in dogs one year of age or younger. There was no difference in the frequency of the autoantibodies based on sex.

In the second population, 14.3% of the dogs had evidence of autoantibodies to Tg, T_4, or T_3. Again, autoantibodies to Tg were present in the highest percentage with 50% of the dogs with autoantibodies being positive. However, in this

population, autoantibodies to T_3 were less common, with the remaining dogs having autoantibodies to T_4 and T_3. The incidence of autoantibodies in this second population of dogs is summarized in Table 3.

In the second population of dogs, the ages ranged from 9 weeks to 8 years of age with the ages of the dogs with autoantibodies ranging from 9 months to 6 years with a mean age of 2.3 ± 2.0 years. No differences between the 2 populations could be determined statistically.

The thyroid hormone concentrations were within the normal range for both populations of dogs. Four dogs in the first study and two dogs in the second study had borderline low hormone concentrations, however these dogs did not have thyroid autoantibodies.

DISCUSSION

The purpose of this study was twofold. The first objective was to establish a baseline for the prevalence of thyroid autoantibodies in a normal population of dogs from a breed predisposed to developing hypothyroidism. The second objective was to perform a preliminary investigation into the prevalence of thyroid autoantibodies in a group of related dogs. It is suprising that autoantibodies to Tg, T_4, and T_3 are found so frequently in dogs with no history of thyroid disease and normal serum thyroid concentrations. This study suggests that the presence of autoantibodies occurs earlier in

thyroid disease before enough of the thyroid gland is destroyed to result in clinical and serological evidence of hypothyroidism. It would be anticipated that the autoantibodies would be present in the serum well before the destruction of the thyroid would affect serum thyroid hormone concentrations. Alternatively, thyroid autoantibodies may not be related to the development of impaired thyroid function.

Thyroid autoantibodies were detected in 12 (36.4%) dogs prior to the first year of age. The presence of autoantibodies in dogs 1 year of age or younger were found to be significant when compared to the general population. However, to understand the biological significance of these autoantibodies in relation to thyroid disease it would be necessary to follow these dogs for several years, to determine progressive changes in circulating thyroid hormone concentrations and changing titers to thyroid autoantibodies.

By working with these populations of dogs in a long term study, the pathogenesis and heritability of autoimmune thyroid disease in the dog could be further studied. The role of the MHC in the dog, dietary iodine, environmental influences and other genetic markers could be studied to develop a better understanding of this complex autoimmune endocrine disease.

TABLE 2.
Distribution of autoantibodies to thyroid antigens in serum samples from 135 Gold Retrievers. Twenty-two (16.3%) of the samples had autoantibodies to Tg, T_4, or T_3.

Antigen	No. of Pos. results	Percent of Pos. results	Percent of All samples
Tg	9	40.9	6
Tg & T_3	1	4.5	0.7
All 3	4	18.2	2.9
T_4	2	9.1	1.5
T_3	6	27.3	4.4

TABLE 3.
Distribution of autoantibodies to thyroid antigens in serum samples from 42 related Gold Retrievers. Six (14.3%) of the samples had autoantibodies to Tg, T_4, or T_3.

Antigen	No. of Pos. results	Percent of Pos. results	Percent of All samples
Tg	3	50	7.1
Tg & T_3	1	16.7	2.4
T_4	1	16.7	2.4
T_4 & T_3	1	16.7	2.4

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ISOLATION OF THYROID PEROXIDASE AND INVESTIGATION OF THYROID PEROXIDASE AUTOANTIBODIES IN THE DOG.

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SUMMARY

Thyroid peroxidase (TPO) was purified from canine thyroid tissue using a modification of the procedure for purifying porcine TPO. The enzyme was solubilized from the membrane using a deoxycholate-trypsin solution followed by ammonium sulfate precipitation and DEAE sephadex chromatography. A monoclonal antibody to canine thyroglobulin (Tg) coupled to a immunoaffinity column was used to eliminate the contaminating Tg from the TPO preparation. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions identified protein bands at 107 kDa and 47 kDa. SDS-PAGE under reducing conditions eliminated the 107 kDa band which was replaced with components at 60, 45 and 39 kDa. Using the TPO preparation as an antigen in a Western immunoblot assay, 50 canine sera were screened for evidence of autoantibodies to TPO. The sera used in this study were from dogs with clinical signs of hypothyroidism and autoantibodies to thyroglobulin, T_4, or T_3. No autoantibodies to TPO were found in any of the sera. Assays were also performed using purified porcine and human TPO and no evidence of cross-reactivity was identified. The absence of autoantibodies to TPO in the dog suggests a different pathogenesis for canine autoimmune thyroid disease than that hypothesized for human lymphocytic thyroiditis.

INTRODUCTION

Thyroid peroxidase (TPO) is a membrane-bound, glycosylated, hemoprotein enzyme that plays an important role in thyroid hormone biosynthesis by catalyzing both the iodination of thyroglobulin (Tg) and the coupling of iodotyrosyl residues in Tg to form thyroxine (T_4) and triiodothyronine (T_3).[1] Purification procedures have been reported for porcine[2], bovine[3], and human TPO.[4]

Sera from human patients with lymphocytic thyroiditis (Hashimoto's disease) usually contain antibodies against a thyroid microsomal antigen. It has been established that TPO is very closely related to, if not identical with, the thyroid microsomal antigen that elicits the production of the serum microsomal autoantibodies.[4]

Lymphocytic thyroiditis in dogs is comparable both histologically and serologically to Hashimoto's thyroiditis in human beings.[5] Several studies in dogs have suggested the presence of microsomal autoantibodies in canine thyroid disease.[6,7] In this study, we developed a method for the purification of canine TPO based on the trypsin-detergent solubilization procedure used to purify porcine TPO.[8]

Using the purified canine TPO and Western blot analysis, we studied canine sera for the presence of autoantibodies to canine TPO and its ability to cross-react with human or porcine TPO. To eliminate contamination with Tg, we passed

the sample through an immunoaffinity column to which a murine monoclonal antibody to canine Tg was adhered.

MATERIALS AND METHODS

Isolation and purification of thyroid antigens

Preparation of Tg Tg was prepared from freshly harvested, flash frozen canine thyroid glands obtained from dogs utilized in a student surgery laboratory. Due to the labile nature of TPO, all procedures were performed on ice or in a cold room unless otherwise noted. The thyroids were homogenized in 0.05 M Tris, 0.4mM KI, pH 7.0, followed by centrifugation at 48,000 G for 1 h. The supernate was used for the Tg preparation, the pellet was further processed for isolation of canine TPO. The supernate containing the Tg was centrifuged at 100,000 G for 1 h and the supernate saved. The Tg was precipitated from the supernate with 1.52-1.76 M ammonium sulfate followed by centrifugation at 20,000 G for 40 min. The pellet was resuspended in phosphate buffered saline (PBS) followed by centrifugation at 100,000 G for 40 min. The supernate was dialyzed for 16 hours in PBS at 4 C and the solution loaded on a Sephycryl S300 column (Pharmacia Fine Chemicals, Piscataway, NJ) and eluted with PBS. The first large peak measured at A 280 was collected and contained the purified Tg.

Isolation and purification of thyroid peroxidase The pellet remaining after the first 48,000 G centrifugation was

homogenized with 0.5mM KI in PBS, pH 7.0. Deoxycholate (1%) was added and the suspension was stirred 12 h at 4 C. The solution was treated with 0.013% trypsin, 0.15 M KCl, 0.3% Triton X-100 and stirred at 25 C for 30 min followed by centrifugation at 100,00 G for 60 min. The pellet was discarded and 50% ammonium sulfate was added to precipitate the protein. Following a 17,000 G centrifugation for 40 min, the soft pellet floating on the top of the tube was retained and re-suspended in a 0.15M KCL Tris HCl, 0.1mM KI solution. This was than centrifuged at 100,000 G for 1 h and the supernate retained. The supernate was dialyzed in 0.025 M Tris-HCl, 0.4 mM KI, pH 7.0 for 16 h. This was then eluted on a DEAE sephadex A-50 column in a step-wise gradient using 0.05, 0.2, and 0.25M KCl. The TPO enzyme activity was collected in a broad peak at 0.2 M KCL Tris HCL of the gradient. The peak fractions were pooled and concentrated in an Amicon ultrafiltration cell. The material from the column was than dialyzed against PBS and loaded on a immuno-affinity column with the Tg murine monoclonal antibody and eluted with PBS, 0.5mM KI, pH 7.0 (see below). The TPO activity was in the fractions in the first peak associated with the flow through of the immunoaffinity column. Protein concentrations were determined by the method of Lowry et al. using bovine serum albumin (BSA) as a standard.[9] The bound thyroglobulin was than eluted from the immunoaffinity column with PBS, pH 2.9.

Other antigens Purified human and porcine TPO were generously supplied by Dr. A Taurog, Department of Pharmacology, University of Texas Health Science Center, Dallas, Texas.

Peroxidase Assay During the purification procedure, the TPO enzyme activity was determined by a modified iodide oxidation assay as described by Nakashima, et al (1978).[10] Briefly, the assay was performed as follows: 2.0 ml of 0.012 M KI in PBS, pH 7.0 were placed in 2 cuvettes. To these were added 50 μl of 5% glucose and 50 μl of sample. The volume in each cuvette was adjusted to 2.1 ml with PBS, pH 7.0 and the cuvettes placed in a Perkin Elmer Lambda 2 double beam spectrophotometer with attached recorder (Okidata, Microline 320). The reaction was initiated in the sample cuvette at room temperature by the addition of 10 μl of 500 μg/ml glucose oxidase and ΔA_{353} was followed for 5 min on the recorder. The ΔA_{353}/min was determined from the linear portion of the chart recording. A straight line was drawn through the points, including the origin, and ΔA_{353}/min per μl of sample was calculated from the slope. A unit of activity is defined as ΔA_{353}/min = 1.0.

In addition, the samples was assayed for the ability of the TPO to catalyze the oxidation of guaiacol to tetraguaiacol.[11] This assay was also performed in the double beam spectrophotometer. Both the sample and the reference cuvette contained 2.0 ml of 66mM guaiacol in PBS, pH 7.0 with

50 μl of sample. The reaction was initiated by the addition of 10 μl of 66 mM H_2O_2 and ΔA_{470} after 60 sec was determined from the chart recording. A unit of activity is defined as ΔA_{470} = 1.0 in 60 sec.

Serum Samples Negative reference sera used in the immunoblots were obtained from 13 dogs with no evidence of autoantibodies to Tg, T_4, or T_3, and normal serum concentrations of thyroid hormones, as determined by radioimmunoassay (RIA); the dogs were euthanatized, and no evidence of lymphocytic thyroiditis was found on microscopic examination of thyroid glands. The fifty canine sera screened in the Western blot technique were obtained from Dr. Jean Dodds, Profession Animal Laboratory and from the Endocrine Laboratory at the Animal Health Diagnostic Laboratory, Michigan State University. All canine sera had autoantibodies to at least one of the three thyroid autoantibodies; Tg, T_4, or T_3. The human serum samples were obtained from 2 Michigan State University Clinical Center patients with Hashimoto's thyroiditis based on commonly accepted clinical and laboratory criteria. One patient had a high microsomal antibody titer and no evidence of Tg autoantibodies while the other sample had high titers to both Tg and the microsomal antigen.

Thyroglobulin monoclonal hybridoma production BALB/c mice were immunized ip with 0.2 mg purified canine Tg in RIBI

adjuvant (RIBI, Hamilton, MT) at 2 week intervals for 8 weeks. The mice were then bled and the serum assayed for anti-Tg antibodies. The mice received a final immunization 3 days prior to cell fusion. Splenic lymphocytes from the immunized mice were fused with P3-X63-Ag8 (CRL 1580) murine myeloma cells using Hyclone Laboratories modification of Fazekas De St. Groth protocol.[12,13] Hybridoma supernates were tested for anti-Tg antibodies using the ELISA assay beginning 14 days post-fusion. The isotype of the positive monoclonal antibodies (mAb), as determined by the Sigma ImmunoType Kit monoclonal antibody test, was IgG2B.

Ascites fluid containing mAb to Tg was obtained from the peritoneal cavity of mice that had been inoculated ip with hybridoma cells. The mAb was purified from the ascites fluid using a protein A-Sepharose CL-4B column with PBS at pH 2.9 to elute off the mAb.

The Tg mAb were concentrated in an Amicon ultrafiltration cell and coupled to Affi-Gel Hz hydrazide gel (Bio-Rad, Richmond, CA) according to manufacturers instructions. Briefly, 0.96 ml of mAb (A_{280} of .983), 96 μl Na perodate (25 mg in 1.2 ml), and 96 μl 0.5 M Na acetate, pH 5.5 were combined and rotated for 1 h at 25 C. The solution was desalted over a Sephadex G 25 column equilibrated with 50 mM Na acetate, 50 mM NaCl, pH 5.5 and fractions 4-9 from a steep peak as detected at A280 were retained. The fractions were concentrated using a Amicon ultrafiltration cell and added to

Affi-Gel Hz hydrazide gel which had been washed with a coupling buffer consisting of 50 mM Na acetate and 50 mM NaCl, pH 5.5, 5 times. The Tg mAb and Affi-Gel Hz were rotated 12 hours at 4 C prior to packing the column. This column was then used to bind and remove the Tg from the TPO preparation.

Enzyme-linked immunosorbent assay for anti-Tg The ELISA was a modification of a procedure described previously.[13] Disposable flat-bottomed polystyrene plates (Corning Glass Works, Corning, NY) were coated with 50 μl of canine Tg (10 μg/ml) in PBS overnight at 4 C. Alkaline phosphatase-conjugated goat antimouse IgG, heavy and light chain, (Sigma) was used as the second antibody. Serum from mice prior to immunization and media from wells without growing hybridomas were used as negative controls. Serum from the mice prior to fusion was used as the positive control. Supernates were considered positive if the absorbance was 2 standard deviations above the average of the negative control.

Gel Electrophoresis and Western Blotting Polyacrylamide electrophoresis (PAGE) under reducing and non-reducing conditions was carried out using the procedure described by Laemmli, using 7.5% cross-linked gel.[14] After electrophoresis, the gel was either stained with Coomassie brilliant blue R-250, or the gel contents were transferred to a nitrocellulose (NC) membrane. The protein bands were electrophoretically

transferred onto NC in 25mM Tris and 200mM glycine buffer, containing 20% methanol in a Bio-Rad Trans Blot Cell. After transfer, NC sheets were incubated overnight in PBS, pH 7.2 containing 5% BSA to block remaining active sites on the NC sheets. The NC sheets were washed twice with PBS with 5% BSA and incubated for 2 h at room temperature with serum sample diluted 1:100 in PBS containing .5% BSA and washed with PBS with 5% BSA. Three different antibody sources were used: 50 canine sera samples positive for autoantibodies to Tg, T_4, or T_3; rabbit anti-porcine TPO antibodies; and human serum from patients with autoantibodies to TPO as described previously. Bound antibody in canine sera was visualized by incubation with horse radish conjugated goat anti-dog IgG (Kirkegaard & Perry, Gaithersburg, MD) reacted with 4-chloro-1-naphthol (4CIN)and H_2O_2. Antibody from the porcine sample was visualized with horseradish peroxidase conjugated rabbit anti-porcine IgG (Sigma) reacted the same as with the dog HRP-IgG. Human antibodies were visualized by incubation for 1 h with [^{125}I]protein A and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

RESULTS

Figure 1 shows results of SDS-polyacrylamide gel electrophoresis under non-reducing conditions (A) and under reducing conditions (B). Only protein staining was used as enzyme activity is lost with treatment with SDS.

Under non-reducing conditions, two bands were observed corresponding to approximately 107 kDa and 47 kDa as determined from simultaneously added markers. The appearance of the bands at 60, 45, and 39 kDa under reducing conditions and the disappearance of the 107 kDa protein suggests that the 107 kDa component is composed of several fragments linked by one or more disulfide bridges.

The enzyme activity of the purified canine TPO is presented in Table 4. A 33 fold purificationof the, as measured by the iodide assay, was attained from the original crude homogenate to the final product following chromatography with the Tg mAb. The trypsin solubilized peroxidase activity was separated by DEAE-sephadex chromatography into fractions with parallel activities toward guaiacol and iodide. After chromatography on DEAE-sephadex, a reduced guaiacol to iodide activity ratio was observed. The higher recovery of iodide activity may be due to the inactivation of some guaiacol active centers or the unmasking of iodide binding sites on the TPO.

The use of the affinity-column eliminated the contaminating Tg without decreasing the specific activity of the TPO. The bands which retained antigenicity toward the Tg-mAb were no longer present when immunoblotting was performed on the gels. The homogeneity of the Tg-mAb was shown by the fact that all of the Tg-mAb was complexed with Tg when the Tg-mAb was mixed with an excess amount of Tg.

Canine sera with autoantibodies to various thyroid antigens including Tg, T_4 or T_3 were immunoblotted against the purified TPO. No evidence of autoantibodies to TPO was observed in the 50 canine sera screened. Additionally, all evidence of the antigenicity of Tg in Tg positive sera was eliminated indicating the success of the affinity-column purification step. There was no evidence of cross-reactivity between the purified canine TPO and either anti-porcine or anti-human TPO antibodies. Additionally, when canine serum was immunoblotted against either porcine or human TPO, there was no evidence of antibodies or cross-reactivity with these perparations of TPO. The porcine and human TPO did react positively in the Western blots using the respective species specific positive serum.

DISCUSSION

Thyroid peroxidase was purified to near homogeneity following solubilization with deoxycholate and trypsin using a combination of differential centrifugation, DEAE-sephadex chromatography and an immunoaffinity column coupled with a mAb to canine Tg. The method used to purify canine TPO was similar to the procedure used by Taurog, et al (1990) to purify porcine TPO.[8] The resulting TPO preparation showed a high specific activity for iodide and guaiacol oxidation. Tryptic digestion of thyroid subcellular components releases a diverse number of peroxidase components depending on the conditions of the digestion. Factors affecting these can

include quality and purity of the trypsin, pH, temperature, length of digestion, and the detergent and solvent treatment of the particles.[15]

The molecular weight of the canine TPO determined by SDS-polyacrylamide gel electrophoresis under non-reducing conditions was 107 kDa with an additional band at 47 kDa. Under reducing conditions three bands were present at 60, 45, and 41 kDa. These results suggest that TPO is a molecule composed of several peptide chains of unequal size. Since dissociation of these chains in SDS occurs after reduction of the protein, we conclude that the chains are covalently cross-linked by disulfide bonds. These results are similar to the molecular weight and structural findings for human and porcine TPO. Kotani, et al (1986) showed human TPO to be present at 100 and 107 kDa region and porcine TPO to be approximately 100 kDa on immunoblots.[16] Taurog, et al (1990) however found TPO at 90 and 60 with SDS-PAGE under reducing conditions and at 93, 36 and 24 KDa under reducing conditions.[17] However, there is enough variation in the molecular weights to suggest possible differences in structure which is confirmed by the lack of cross-reactivity between canine TPO and either human or porcine TPO. In addition, the use of trypsin to solubilize the thyroid membranes may have eliminated or changed the various components of the canine TPO molecule. It has been documented that trypsin-detergent solubilization procedure cleaves sites at the amino and carboxyl ends of TPO and an

internal site of both human and porcine TPO.[8,17] Thus the use of trypsin for the solubilization may have affected epitopes within the canine TPO molecule. However, previous findings have shown that autoantibodies to TPO are heterogenous in nature so it is unlikely that all antigenicity of the molecule was destroyed in the purification procedures.[18]

The immunoaffinity chromatography method used in this study for the purification of TPO eliminated the contaminating Tg. Since it has been previously shown that dogs have autoantibodies to Tg, the elimination of this antigen improved our ability to investigate possible autoantibodies to canine TPO.[7,13] The relationship of thyroid autoantibodies to Tg, T_4, or T_3 in canine serum to canine lymphocytic thyroiditis has been previously investigated.[14] These studies have indicated that there is a high incidence of autoimmune thyroiditis in dogs that have evidence of these autoantibodies. In the present study we used canine sera with these autoantibodies to investigate autoantibodies to canine TPO. Previous studies had indicated that autoantibodies to a microsomal antigen may be present in dogs, however no confirming evidence was found in this study.[6,7] Previous studies which had identified microsomal autoantibodies in the dog were based on a complement fixation assay using a crude thyroid extract or indirect immunofluorescence. The presence of microsomal autoantibodies was based on the theory that Tg autoantibodies do not fix complement so positive results were indicative of

microsomal autoantibodies which have been shown to fix complement in human sera.[19] The indirect immunofluorescence methods used were discarded as they were found to be technically unreliable and inconsistent. This study used purified TPO as an antigen and sensitive immunoblotting procedures to search for autoantibodies to TPO in the dog. No evidence of autoantibodies to TPO were found. The lack of autoantibodies to TPO is similar to results seen in the obese strain of chicken and rats with lymphocytic thyroiditis (personal communication). The absence of autoantibodies to TPO in the dog suggests that canine lymphocytic thyroiditis has a different pathogenesis than found in human thyroid disease.

TABLE 4.
Activity of canine TPO at various stages of purification.

Purification	Total Protein[a] (mg)	Specific Activity Guaiacol Oxidation	Specific Activity I_3 Formation
1. Ammonium sulfate precipitation	57.11	0.61	0.83
2. DEAE sephadex chromatography	4.20	2.15	1.32
3. Immunoaffinity chromatography[b]	0.78	19.80	4.35

a Based on Lowry protein determination
b Canine Tg mAb coupled to Affi-Gel Hz

FIGURE 5.

SDS-polyacrylamide gel electrophoresis of purified canine TPO stained with Coomassie blue. Lane (a) gel was electrophoresised under non-reducing conditions and lane (b) was electrophoresised under reducing conditions. Approximately 50 μg of protein was applied to gel.

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STUDIES OF AUTOANTIBODIES TO CANINE TPO BY ENZYME-LINKED IMMUNOSORBENT ASSAY AND INHIBITION OF ENZYME ACTIVITY

SUMMARY

Serum autoantibodies to the microsomal antigen which has been identified as thyroid peroxidase (TPO) are common in human patients with autoimmune thyroid diseases. Autoantibodies to TPO are important in the diagnosis of autoimmune thyroid disease in human beings as greater than 90% of these patients have autoantibodies to this thyroid antigen.

Two studies in dogs found 'microsomal' autoantibodies in dogs with thyroid disease.[1,2] These studies used either complement fixation and an ELISA using a crude thyroid preparation or indirect immunofluorescence on frozen and fixed canine thyroid glands. Because of the nature of these assays, we decided to develop a sensitive ELISA for the detection of autoantibodies to TPO in the dog.

Studies of TPO autoantibodies in human beings indicate the presence of TPO autoantibodies in the serum inhibits the enzyme activity of TPO.[3] Using purified canine TPO, canine sera were tested for TPO autoantibodies by assessing their ability to inhibit the enzyme activity of TPO.

The objective of this study was to develop assays to detect autoantibodies to TPO in dog. Hypothyroidism is a commonly diagnosed endocrine disease in dogs. However, the

accurate diagnosis of hypothyroidism is often difficult due to the many factors which affect thyroid hormone concentrations. The development of assays to detect and measure autoantibodies to TPO will potentially aid veterinary practitioners in diagnosing canine autoimmune thyroiditis.

MATERIAL AND METHODS

Serum Samples Negative control serum was obtained from dogs which had normal serum thyroid hormone concentrations as diagnosed by RIA, no detectable autoantibodies to TG, T_4, or T_3 and upon euthanasia their thyroid glands had no microscopic evidence of thyroid disease. The canine sera screened for evidence of autoantibodies to TPO was from dogs with autoantibodies to TG, T_4, or T_3 as documented by assays performed in our laboratory.

Preparation of canine TPO The purification of TPO used in this project was described in chapter V. Two preparations of TPO were used in the ELISA. The first preparation consisted of TPO which had been isolated by differential centrifugation and DEAE-sephadex chromatography, but had not been eluted through the immunoaffinity column bound with the Tg mAb. The second preparation consisted of TPO from the first preparation which had been eluted through the Tg immunoaffinity column prior to using in the ELISA. The inhibition studies used only TPO which had not been run through the immunoaffinity column

as Tg autoantibodies should not interfere with the TPO enzyme activity.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was a modification of the assay used for the measurement of Tg autoantibodies as described previously. Briefly, 100 µl of antigen solution was added to the wells of a microtiter plate at the following concentrations: TPO 20 µg/ml diluted in 50 mM Carbonate buffer, pH 8.5 and Tg, 10 µg/ml in PBS, pH 7.4 and incubated overnight at 4 C. Canine serum was diluted 1:100 with PBS with 0.5% BSA and incubated for 2 h at 25 C. Alkaline phosphatase-conjugated goat anti-canine immunoglobulin G (heavy and light chain) (Kirkegaard & Perry, Gaithersburg, MD) was added following washing the plates with 5% BSA in PBS, pH 7.4 and incubated for 1 h at 25 C. The secondary antibodies were reacted with the enzyme substrate, p-nitrophenyl phosphate (1 mg/ml) and the absorbance was read on a spectrophotometer at 405 nm.

Inhibition of TPO catalytic activity by patient serum

Purified canine TPO with a specific activity of 21.43 U/mg protein as determined by the iodide oxidation assay described earlier was used in the assay. Fifty µl of TPO was incubated with 50 µl of patient sera or negative control sera and incubated 1 h at 25 C. For the measurement of the catalytic activity, 2.0 ml PBS with 0.012 M KI pH 7.0 and 50 µl 5%

glucose to 2 cuvettes, 50 μl of the incubated sample was added to 1 of the cuvettes. The reaction was initiated by the addition of 10 μl of 500 μg/ml glucose oxidase and read in a Perkin Elmer Lambda 2 double beam spectrophotometer connected to a Okidata Microline 320 reader. The ΔA353/min was determined from the linear portion of the chart recording. A straight line was drawn through the points, including the origin, and ΔA353/min per μl of sample was calculated from the slope. A unit of activity is defined as ΔA353/min = 1.0.

In one experiment, the effect of addition of varying amounts of serum to the iodide oxidation assay was determined. Incubation and assay were performed as described above. For each volume of patient serum added, results were compared with a similarly treated control serum.

To confirm that autoantibodies to Tg were not interfering with the catalytic activity of the TPO, serum was incubated with excess Tg to absorb out all Tg autoantibodies.

SDS-polyacrylamide electrophoresis and immunoblotting This procedure was explained in the chapter on the purification of TPO. Purified canine TPO, Tg and trypsinized Tg were electrophoresed on SDS-polyacrylamide gels and immunoblotted as previously described. The proteins were transferred to a nitrocellulose sheet which was incubated with negative control canine sera, serum from 2 dogs with documented autoantibodies to Tg, and the Tg mAb described in chapter V.

RESULTS

Binding of the canine serum to the canine TPO antigen preparation occurred in 5 of the 39 canine serum samples screened in the ELISA. However, when purified canine Tg was incubated with the serum samples to absorb out all autoantibodies to canine Tg, all samples became negative. To confirm the presence of Tg contamination in the initial purified canine TPO, the TPO was immunoblotted along with purified canine Tg and trypsinized canine Tg. The immunoblotting confirmed the contamination of the TPO with Tg. After purifying the TPO further using immunochromatography with the mAb to canine Tg, all evidence of the contaminating Tg disappeared on SDS-polyacrylamide gel electrophoresis and immunoblotting. When this TPO was used as the antigen in the ELISA, no positive results were evident.

Because inhibition of the catalytic activity of TPO had been demonstrated with serum from human patients with autoimmune thyroid disease, we repeated the assay using the iodide oxidation assay and canine serum. Consistent inhibition of TPO activity by patient sera in relation to negative control sera did not occur.

DISCUSSION

No autoantibodies to canine TPO were detected using an ELISA or an assay for the inhibition of enzyme catalytic activity. Initially, in the ELISA using the first TPO

preparation, 5 serum samples appeared to have evidence of TPO autoantibodies. However, the positive results were the result of a small amount of Tg which was contaminating the TPO sample. Because it has been documented dogs with thyroid disease have autoantibodies to Tg, it was not surprising that dogs with very high titers to the Tg appeared positive in this ELISA[4]. To confirm that the positive results seen in our assay were to Tg, the autoantibodies were absorbed with excess Tg. The serum samples than became negative in the ELISA. The presence of Tg in the TPO preparation was confirmed using SDS-polyacrylamide electrophoresis and immunoblotting. Because the addition of a large amount of Tg might have an effect other aspects of the ELISA it was necessary to eliminate the contaminating Tg.

To eliminate the contaminating Tg, the TPO preparation was eluted through an immunoaffinity column that contained Tg mAb coupled to the gel. The resulting preparation had no evidence of Tg as shown by repeating the SDS-polyacrylamide gel electrophoresis and immunoblotting. When this purified TPO was used as the antigen in the ELISA, no sera samples were positive. To investigate whether canine autoantibodies to TPO may inhibit the catalytic activity of the enzyme, purified TPO was incubated with serum. Because the presence of Tg autoantibodies should not interfere with the activity of the TPO, we used the initial TPO preparation. No sera consistently inhibited the activity of the TPO as measured by

iodide oxidation. To determine that Tg autoantibodies did not play a role in this lack of inhibition, the Tg autoantibodies were again absorbed out with Tg. No change in results was seen following the depletion of the Tg autoantibodies. We would not expect to see any inhibition of TPO activity by canine serum since no evidence of TPO autoantibodies were found using the more sensitive ELISA.

The previous study that reported the presence of microsomal autoantibodies in 1 dog was an ELISA used a very crude thyroid extract as antigen.[2] It is our contention that this dog reacted to the Tg which was present in the antigen. Thyroglobulin autoantibodies can be present in very high titer (>24,000) and thus it is difficult to absorb out all the Tg autoantibodies using excess Tg without diluting out the serum sample. On the basis of these results, we concluded that the incidence of autoantibodies to TPO was either below the sensitivity of these assays, or very low or dogs do not produce autoantibodies to TPO.

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SUMMARY AND CONCLUSIONS

The objective of this study was to develop assays to detect autoantibodies to the thyroid antigen, Tg, T_4, T_3, and TPO in the dog. An ELISA was developed to detect and measure autoantibodies to canine Tg. An agarose gel electrophoresis assay was used for the measurement of autoantibodies to canine T_4 and T_3. Using these assays, a study was performed on serum from dogs (n=119) with clinical symptoms of hypothyroidism including alopecia, lethargy and obesity. Autoantibodies to Tg, T_4, or T_3 were found in 48.7 percent of the samples measured. Autoantibodies to Tg were found most frequently (42%) and were statistically related to low serum T_4 hormone concentrations. In addition, autoantibodies to T_4 or T_3 were found in 26.1% and 32.8% of the samples respectively. Of the positive samples 32.7% had autoantibodies to all 3 antigens.

Using the assays to detect serum Tg, T_4, and T_3 autoantibodies, a study was done on 2 populations of Golden Retrievers. The first population (n=135) was from clinically normal dogs. In this population, autoantibodies to one of the 3 antigens were found in 16.3% of the dog. A significantly higher percentage of thyroid autoantibodies were present in dogs less than 1 year of age. The serum thyroid hormone concentrations of these dogs were normal and only 2 dogs had slight clinical signs of lethargy or obesity. The percentage and the presence of these autoantibodies in young dogs indicates these assays may be helpful in early diagnosis of

autoimmune thyroid disease in dogs when coupled with resting serum T_4 or T_3 concentrations.

The second population of Golden Retrievers consisting of dogs related to a dog from the first population which had autoantibodies to Tg, T_4, and T_3 was examined to determine if there was a familial basis to thyroid autoantibody development. In this population, 14.3% of the dogs had autoantibodies to the various thyroid antigens. Again, 66.6% of the dogs with autoantibodies were less than 1 year of age. The fact that the percentages of autoantibodies in this population was similar to the general population suggests that while heritability may be a factor, it cannot be considered the sole initiating factor in the development of thyroid autoantibodies. Further studies should be done to confirm this observation.

Canine TPO was isolated and purified using a modification of a technique developed for porcine TPO using differential centrifugation and DEAE-sephadex chromatography. An ELISA using semi-purified canine TPO was used to screen sera for the presence of autoantibodies. Of the 39 canine serum assayed 5 appeared to be positive. Upon further investigation this preparation was found to be contaminated with Tg which interfered with the testing for TPO autoantibodies as the Tg autoantibodies present in the serum resulted false positive results. To eliminate the contaminating Tg from the TPO, a monoclonal antibody (mAb) to canine Tg was made. The Tg mAb

was coupled to an immunoaffinity column and the canine TPO was eluted through the column removing the Tg.

When all Tg was removed from the TPO preparation, as confirmed by SDS-polyacrylamide gel electrophoresis and immunoblotting with canine sera containing autoantibodies to TG, there was no indication of autoantibodies to TPO in the ELISA. To confirm this assumption, a sensitive Western blot assay was developed. Canine serum with autoantibodies to other thyroid antigens including Tg, T_4 and/or T_3 were screened using the Western blot assay for evidence of autoantibodies to canine TPO. No evidence of autoantibodies were found in any of the samples. Additionally, an assay to investigate the inhibition of TPO's catalytic activity by TPO autoantibodies using the iodide oxidation assay was inconsistent and showed no reliable data to support the existence of TPO autoantibodies.

On the basis of our study of thyroid antigens and autoantibodies, it appears that Tg plays a major role in canine autoimmune thyroid disease. In the study of Tg, T_4, and T_3 autoantibodies, it was apparent that the presence of autoantibodies to Tg are significant in relation to clinical signs of hypothyroidism and decreased serum concentrations of T_4. Additionally, autoantibodies to T_4 and T_3 may be a subset of Tg autoantibodies. Thyroid hormones are haptens and thus alone cannot induce antibody formation. Thyroglobulin contains many T_4 and T_3 molecules to which autoantibodies can

be produced. However, autoantibodies to T_4 and T_3 occasionally were present without Tg autoantibodies suggesting that the thyroid hormones may occasionally bind to other serum proteins to induce antibody production. However, when thyroid hormone autoantibodies were present without Tg autoantibodies, they were not statistically significant in relation to serum T_4 concentrations. This suggests that when they are present alone, it may be more indicative of changed serum binding than thyroid disease. However this should be further investigated.

Canine TPO was isolated and used in this study to determine if dogs had autoantibodies to this antigen. Because of the frequency of autoantibodies to Tg, it was necessary to eliminate this antigen as a source of contamination of our TPO. Because the quantity of TPO was small, we used a Western blots as opposed to the less sensitive ELISA procedure to examine sera for TPO autoantibodies. When all Tg was eliminated from the TPO, no autoantibodies to TPO were present in the samples examined. In addition, TPO and serum from normal or potentially hypothyroid dogs did not cross-react with either TPO or anti-TPO serum from pigs or human beings. Thyroid peroxidase is an important antigen in the pathogenesis of human thyroid disease. It is felt that the combined expression of TPO and the inappropriate expression of class II MHC antigens on thyroid epithelial cells play a role in the initiation of autoimmune thyroid diseases in human beings. This study, by failing to detect autoantibodies to TPO

indicates a different pathogenesis and different initiating factors may be present in canine thyroid disease.

Because the accurate diagnosis of hypothyroidism in dogs is an important concern to pet owners and dog breeders alike, it is imperative that we learn more about this disorder. The thyroid is one of the most important endocrine organs in the body and its function is affected by many outside influences. Influences which alter the thyroid hormone concentrations also make the diagnosis of hypothyroidism difficult. Additionally, it is a disorder that tends to affect dogs in their middle years, so many reproduce prior to the discovery of autoimmune thyroid disease. Because the genetic heritability of autoimmune lymphocytic thyroiditis has been documented in some breeds of dog, an accurate early diagnosis would be useful to selectively breed dogs without incipient thyroid disease.

Continued investigation of Tg is necessary to determine its exact role in the initiation of autoimmune thyroid disease. Some aspects to investigate are:

1. Continue measuring the thyroid hormone concentrations and the thyroid autoantibodies in the young Golden Retrievers to determine whether the presence of thyroid autoantibodies in young, normal dogs is a diagnostic tool for determining dogs at risk for autoimmune thyroiditis and clinical hypothyroidism.

2. The role lymphocytes play in the destruction of the thyroid gland. It needs to be determined whether thyroid parenchymal destruction in canine lymphocytic thyroiditis is primarily B or T lymphocyte mediated disorder.

3. If a change in the structure of Tg plays a role in the induction of thyroid disease. In the obese strain of chickens, which are susceptible to autoimmune lymphocytic thyroiditis, increased iodination plays a major role in increased Tg antigenicity. Studies could be done using the mAb to Tg developed in this project to investigate the structure and location of the Tg in the thyroid.

4. Investigate the Tg gene to see if there is a variation in the sequence in normal and affected dogs.

5. Family studies, including investigation of the MHC in dogs and the possible role it may play in the pathogenesis of thyroid disease.

5. Investigate whether dogs have other thyroid autoantibodies, e.g. thyroid enzyme inhibiting autoantibodies.

At this time, the initiating event which triggers the beginning of the destruction of the thyroid by lymphocytes is

unknown. Although there is a genetic predisposition, we may find that there are other environmental and dietary factors play a major role in its pathogenesis.