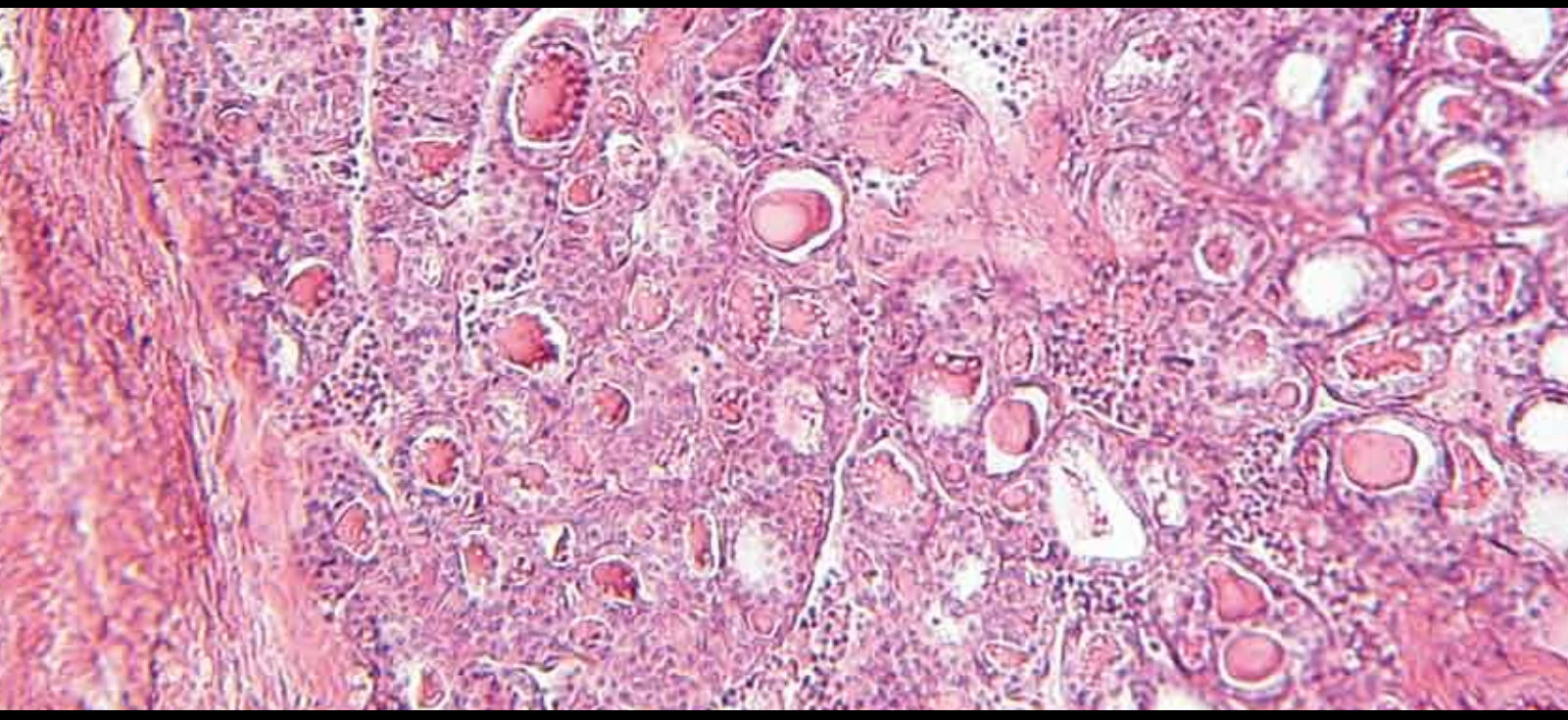
Journal of Thyroid Research

Autoimmune Thyroid Disorders

Guest Editors: Rosalind Brown and Gary L. Francis



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Editorial

Autoimmune Thyroid Disorders

Rosalind Brown[1] and Gary L. Francis[2]

[1] *Pediatrics, Harvard Medical School, Boston, MA 02115, USA*
[2] *Section of Pediatric Endocrinology and Metabolism, Children's Hospital of Richmond at the Virginia Commonwealth University Health System, P. O. Box 980140, Richmond, VA 23098, USA*

Correspondence should be addressed to Gary L. Francis, glfrancis@vcu.edu

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Thyroid autoimmunity, as reflected by the presence in serum of autoantibodies directed against the thyroid autoantigens thyroglobulin (Tg) and thyroid peroxidase (TPO), is present in >10% of the US population over 12 years of age [1] and is the most common cause of endocrine dysfunction in iodine-sufficient populations [2]. The underlying mechanism is a failure of T-cell tolerance leading to lymphocytic infiltration of the thyroid gland [3] and to a complex sequence of humoral and cellular immune responses to thyroid antigens, presumably in response to an environmental trigger [4]. In chronic lymphocytic thyroiditis (CLT), the predominant immunologic mechanisms are T-cell- and cytokine-mediated thyroid cell damage and apoptotic cell death whereas in Graves' disease (GD) generation of thyrotropin (TSH) receptor autoantibodies leads to thyroid cell stimulation [5], but significant overlap exists. Seven susceptibility genes, in addition to the major histocompatibility gene (HLA-DR3), have now been identified [6]. Some of these genes affect the immune response in general (CD40, CTLA-4, and PTPN22), while others are thyroid specific (thyroglobulin, thyrotropin (TSH) receptor). Some are common to both CLT and GD, while others are specific for GD.

In view of the importance of AITD as well as the diverse array of new information, it is only fitting that this special issue of the Journal of Thyroid Research is devoted entirely to this complex subject. Four of the papers we have selected are focused on clinical topics, including AITD in childhood, during pregnancy, in the postpartum period, and in patients with type 1 diabetes mellitus. The fifth paper addresses the potential role of NKT cells in an animal model of thyroiditis.

We conclude this special edition with a discussion of thyroid autoimmunity in patients with papillary thyroid cancer (PTC). The association of AI and thyroid cancer was first reported by Dailey et al. [7]. In general, patients with AI appear more likely to have PTC than follicular thyroid cancer (FTC), but a lower frequency of extrathyroidal extension, nodal and distant metastases when compared with patients without AI. In some but not all series, patients with autoimmune thyroiditis (AT) and PTC have improved survival when compared to those with PTC alone, suggesting that thyroid autoimmunity might contribute to improved survival [8–10]. In contrast, other data suggest that AI might actually increase the risk to develop thyroid cancer [10–12].

Several theories have been proposed to explain how AI might increase the risk for thyroid malignancy. Thyrocyte apoptosis and proliferation are increased in AI suggesting that thyrocytes rapidly progressing through the cell cycle might accumulate increased DNA damage resulting in malignant transformation [13]. Russell et al. hypothesized that thyroid cells predestined to become cancers might secrete proinflammatory cytokines that affect immune cells [14]. They showed that thyrocytes of ret/PTC3 transgenic mice express increased levels of interleukins, tumor necrosis factor-α, and cyclooxygenase-2 [14] that could attract and/or activate cells of the immune system. Finally, the ret/PTC recombinant genes have been detected in samples of AI [15–17] suggesting that ret/PTC rearrangements might be present in AI and could be precursors to PTC.

From these papers, it is clear that thyroid autoimmunity is a frequent problem in the population and that thyroid autoimmunity can lead to a variety of thyroid disorders including alterations in thyroid hormone synthesis and possibly even neoplasia. Focused research in this area is

beginning to illuminate some of the molecular mechanisms that help to explain these associations.

Rosalind Brown
Gary L. Francis

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Review Article

Autoimmune Thyroid Diseases in Children

Marco Cappa, Carla Bizzarri, and Francesca Crea

Unit of Endocrinology and Diabetes, Bambino Gesù Children's Hospital, University of Rome "Tor Vergata", Piazza S. Onofrio 4, 00165 Rome, Italy

Correspondence should be addressed to Marco Cappa, cappa@opbg.net

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The two major autoimmune thyroid diseases (ATDs) include Graves' disease (GD) and autoimmune thyroiditis (AT); both of which are characterized by infiltration of the thyroid by T and B cells reactive to thyroid antigens, by the production of thyroid autoantibodies and by abnormal thyroid function (hyperthyroidism in GD and hypothyroidism in AT). While the exact etiology of thyroid autoimmunity is not known, it is believed to develop when a combination of genetic susceptibility and environmental encounters leads to breakdown of tolerance. It is important to recognize thyroid dysfunction at an early stage by maintaining an appropriate index of suspicion.

1. Introduction

Autoimmune thyroid disease (ATD) is the most common autoimmune condition, affecting approximately 2% of the female population and 0.2% of the male population [1]. Its overall prevalence peaks in adulthood; it is also the most common etiology of acquired thyroid dysfunction in paediatrics. It is more common in females and usually occurs in early to mid-puberty [2, 3]. Optimal quantities of thyroid hormone are critical to neurodevelopment and growth. The paediatrician can often recognize thyroid dysfunction in its early stages, by maintaining an appropriate index of suspicion.

This review will analyze current opinions and options regarding the etiology, evaluation, diagnosis, treatment, and prognosis of ATDs in children.

1.1. Etiology. ATD arises due to complex interactions between environmental and genetic factors, that are yet to be completely defined. ATD is multifactorial in that a genetic predisposition combines with environmental risk factors to promote disease.

Early evidence that ATD has a hereditary component stems from studies of familial aggregation. Several studies of young people with ATDs showed a definite genetic propensity for thyroid autoimmunity to run in families [4]. Further evidence of the genetic control of ATDs comes from the observation of twins. Monozygotic twins show a higher concordance rate of disease than dizygotic twins. However, even with identical twins the concordance rate is only about 50%, emphasizing that other important factors, such as the environment, play a role in disease pathogenesis [5–7]. The identified ATDs susceptibility genes can be divided into two broad groups:

(1) immune modulating genes, and

(2) thyroid specific genes.

The immune modulating genes so far identified are**:** HLA-DR, CTLA-4, CD40, and PTPN22. The cytotoxic T lymphocyte-associated factor 4 (CTLA-4) gene is a major negative regulator of T-cell activation [8]. CTLA-4 activation has been shown to suppress several experimental autoimmune diseases. CD40 [9] is expressed primarily on B cells and other antigen presenting cells (APCs) and plays a fundamental role in B-cell activation inducing, upon ligation, B-cell proliferation, immunoglobulin class switching, antibody secretion, and generation of memory cells. The lymphoid tyrosine phosphatase, encoded by the protein tyrosine phosphatase-22 (PTPN22) gene, like CTLA-4, is a powerful inhibitor of T-cell activation [10]. Recently, linkage studies mapped ATDs susceptibility loci in two thyroid specific genes, the thyroglobulin (TG) [11] and TSH receptor (TSHr) genes [12], that represent the main targets of the immune response in ATDs.

Polymorphic variations of all the cited genes have been identified and linked to ATDs susceptibility, but the existing studies have often given inconsistent results, with some showing associations and others not. One of the many unexpected findings of these genetic studies is that most of the identified genes have a very minor effects. Indeed, with the exception of the DRb1-Arg74 HLA variant, which gave an odd ratio for Graves' disease (GD) of >5, all the other ATDs genes gave very low odd ratios of <1.5 [13]; on the other hand, family history is positive in about 50% of patients with ATDs. It is usually supposed that a strong genetic effect on disease is related to the inheritance of many genes with small effect. Two alternative mechanisms have been proposed for the finding of very low odd ratios for most ATDs genes [4]: subset effect and gene-gene interactions. According to the "gene-gene interaction" model, two genes with weak effects (i.e., associated with low odd ratios) interact, biologically resulting in a combined odd ratio that is significantly higher than the one expected with an additive effect alone. For example, two genes with odd ratios for disease of 1.2 when inherited together would give an odd ratio of 1.44 (1.2×1.2), if there was only an additive effect. If there is an interaction between these two genes, the odd ratio for disease will be significantly higher. According to the "subset effect" model (also called genetic heterogeneity), each of the genetic variants identified has a large effect resulting in a high odd ratio in a subset of the ATDs patients studied. On the contrary, when these variants are tested in the entire population of ATDs patients, their effects are diluted, resulting in much smaller odd ratios.

A recent twin study estimated that 79% of the liability to the development of GD is attributable to genetic factors [14]. Therefore, about 20% of the liability to develop GD is due to nongenetic factors. Among the nongenetic factors postulated to precipitate ATDs are iodine [15, 16] and medications such as amiodarone [17] and interferon α [18], infections, smoking, and stress. Amiodarone is a benzofuranic-derivative iodine-rich drug widely used for the treatment of tachyarrhythmias. It often causes changes in thyroid function tests (typically an increase in serum T_4 and rT_3 and a decrease in serum T_3 concentrations), mainly related to the inhibition of 5′-deiodinase activity. In 14–18% of amiodarone-treated patients, there is overt thyroid dysfunction, either amiodarone-induced thyrotoxicosis (AIT) or amiodarone-induced hypothyroidism (AIH). Both AIT and AIH may develop either in apparently normal thyroid glands or in glands with preexisting, clinically silent abnormalities. Preexisting Hashimoto's thyroiditis is a definite risk factor for the occurrence of AIH. The pathogenesis of iodine-induced AIH is related to a failure to escape from the acute Wolff-Chaikoff effect due to defects in thyroid hormonogenesis and, in patients with positive thyroid autoantibody tests, to concomitant Hashimoto's thyroiditis. AIT is primarily related to excess iodine-induced thyroid hormone synthesis in an abnormal thyroid gland (type I AIT) or to amiodarone-related destructive thyroiditis (type II AIT), but mixed forms frequently exist [17].

A few studies have shown seasonality [19, 20] and geographic variation [21] in the incidence of GD, adding evidence that infectious agents may trigger ATDs. Moreover, several infectious agents have been implicated including Yersinia enterocolitica [22, 23], Coxsackie B virus [24], retroviruses [25, 26], and Helicobacter pylori [27].

By now, the strongest association of ATDs with an infectious agent is with hepatitis C virus (HCV) [28]. In most studies examining the frequency of thyroid disorders in hepatitis C patients, approximately 10% of the patients had positive autoantibodies prior to initiation of interferon therapy [29, 30]. Pooling of data from all studies on HCV infection and thyroid autoimmunity demonstrated a significant increase in the risk of ATDs in HCV patients [31].

Two main theories have been proposed for the induction of autoimmunity by infectious agents: (1) the "molecular mimicry" theory suggests that sequence similarities between viral or bacterial proteins and self proteins can induce a cross-over immune response to self antigens [32]; (2) the "bystander activation" theory proposes that viral infection of a certain tissue can induce local inflammation and cytokine release, resulting in activation of autoreactive T cells, that were suppressed by peripheral regulatory mechanisms [33].

2. Autoimmune Thyroiditis (AT)

The childhood prevalence of chronic autoimmune thyroiditis (AT) peaks in early to mid-puberty, and a female preponderance of 2 : 1 has been reported [34]. Presentation is rare under the age of 3 years, but cases have been described even in infancy [35].

2.1. Terminology and Definitions. In 1912, Hashimoto described four women with goiter and the apparent transformation of thyroid into lymphoid tissue (struma lymphomatosa). These patients comprise the first report of Hashimoto's disease, which we now recognize as a form of AT. Improvements in the measurement of circulating autoantibodies and ultrasonography have obviated the need for biopsy in the diagnosis of AT. The term thyroiditis is defined as evidence of "intrathyroidal lymphocytic infiltration" with or without follicular damage. Two types of AT (also defined as chronic lymphocytic thyroiditis) are causes of persistent hypothyroidism: Hashimoto's disease (goitrous form) and atrophic thyroiditis (nongoitrous form). Both are characterized by circulating thyroid autoantibodies and varying degrees of thyroid dysfunction, differing only by the presence or absence of goiter. Transient thyroiditis seems to be a variant presentation of AT. It is characterized by an autoimmune-mediated lymphocytic inflammation of the thyroid gland resulting in a destructive thyroiditis with release of thyroid hormone and transient hyperthyroidism, frequently followed by a hypothyroid phase and full recovery. The condition is particularly common in the postpartum period, but it has been observed also in children. The term chronic AT does not include subacute (de Quervain's) thyroiditis.

2.2. Pathophysiology. The activation of CD4 (helper) T-lymphocytes specific for thyroid antigens is believed to be the first step in pathogenesis. Once activated, self-reactive CD4

T cells recruit cytotoxic CD8 T cells as well as autoreactive B cells into the thyroid. The three main targets of thyroid antibodies are thyroglobulin (TG), thyroid peroxidase (TPO), and the TSH receptor (TSHr). Anti-TPO antibodies have been shown to inhibit the activity of the enzyme in vitro, but direct cytotoxicity by CD8 T cells is believed to be the main mechanism of hypothyroidism in vivo. Anti-TSHr antibodies of the blocking type may contribute to hypothyroidism in a minority of adult patients with the atrophic form of AT, but this has not been proven in children. Histologically, goitrous AT is characterized by diffuse lymphocytic infiltration with occasional germinal centers. Thyroid follicles may be reduced in size and contain sparse colloid. Individual thyroid cells are often enlarged with oxyphilic cytoplasm (usually defined Hurthle cells). In contrast, the gland of atrophic AT is small, with lymphocytic infiltration and fibrous replacement of the parenchyma [36].

2.3. Clinical Aspects. AT is usually suspected in the presence of goiter, even in the absence of signs and symptoms of thyroid dysfunction. It may also be diagnosed incidentally during medical checkups, screening evaluation of children with growth defects, or followup of children with associated diseases, mainly Down syndrome, Turner syndrome, type 1 diabetes, and celiac disease [37–39]. Additionally, a recent study [40] analyzed morpho-volumetric and functional thyroid abnormalities in young patients with Williams syndrome: 31.5% had subclinical hypothyroidism with TSH above the upper normal limit and normal FT_3 and FT_4 concentrations, and 67.5% had morphological or volumetric abnormalities of the thyroid gland at ultrasonography. Anti-TPO antibodies and anti-TG antibodies were absent in all patients, suggesting embrional defect of thyroid morphogenesis and/or a delayed maturation of the hypothalamic-pituitary-thyroid axis, instead of an ATD, more common in the other syndromes.

In all patients with associated diseases, AT is usually detected in its initial phase when thyroid function is preserved, with normal or only slightly elevated TSH levels. At this stage, signs and symptoms of thyroid disease are usually absent, but because worsening of thyroid function is a possibility, early recognition of thyroid dysfunction is necessary to prevent the negative effects of hypothyroidism on growth and metabolic function. The enlarged thyroid gland usually is diffuse and nontender; sometimes the gland may be firm [36]. As the disease progresses, subclinical and then clinical hypothyroidism appears. Symptoms of hypothyroidism may be subtle, even with marked biochemical derangement (Table 1). The initial history should investigate energy level, sleep pattern, menses, cold intolerance, and school performance. In addition to palpation of the thyroid, assessment of the extra ocular movements, fluid status, and deep tendon reflexes are important components of the physical examination. AT may be the initial presentation of an autoimmune polyglandular syndrome, and the possibility of coexisting autoimmune diseases such as type 1 diabetes, celiac disease, Addison's disease, and pernicious anemia must be addressed by the past medical history. Screening for other autoimmune diseases should be undertaken if clinically indicated. Growth and pubertal development may be deranged. Similar to other endocrine causes of growth failure, linear growth is compromised to a greater degree than weight gain, and the bone age is delayed [41, 42]. Hypothyroidism typically causes pubertal delay but may also induce pseudoprecocious puberty, manifested as testicular enlargement in boys, breast development, and/or vaginal bleeding in girls [43–45]. This syndrome clinically differs from true precocity by the absence of accelerated bone maturation and linear growth.

Table 1: Symptoms and signs of overt hypothyroidism.

Goiter
Poor linear growth with increased weight for height
Bone maturation delay
Pubertal disorders (pubertal delay or pseudoprecocious puberty)
Irregular menstrual periods
Lethargy and/or impaired school performance
Fatigue
Bradycardia and decreased cardiac output
Constipation
Cold intolerance
Hypothermia
Fluid retention and weight gain (due to impaired renal free water clearance)
Puffiness of the face
Dry skin
Increased body hair
Delayed relaxation phase of the deep tendon reflexes

2.4. Diagnosis. The serum TSH concentration is elevated in primary hypothyroidism and its determination is an appropriate screening test for thyroid dysfunction. If the differential diagnosis includes central hypothyroidism or if the overall suspicion for overt hypothyroidism is high, FT_4 should be included. In mild hypothyroidism, serum FT_3 can remain in the normal range due to the increased conversion of FT_4 to FT_3 by type 2 deiodinase and the preferential secretion of FT_3 by residual thyroid tissue under the influence of high TSH levels [46]. For these reasons, measurement of the serum T_3 and FT_3 concentration is not a useful test in the diagnosis or monitoring of patients with primary hypothyroidism. The presence of goiter or high TSH levels should prompt the measurement of anti-TPO antibodies. Anti-TPO antibodies are the most sensitive screen for AT. Little further benefit is gained by the additional measurement of anti-TG antibodies, although they may be added if anti-TPO titers are negative [47]. Ultrasonography of the gland shows characteristic structural abnormalities such as generalized hypoechoicity and disomogeneity, due to inflammation and diffuse lymphocytic infiltration with occasional germinal centers (pseudonodules). A diffuse

fibrosis of the gland can become evident at a later stage of the disease [48].

We recommend thyroid ultrasound to confirm AT diagnosis and to investigate the appearance of thyroid nodules during followup, although it is not considered as a standard of care.

The typical patient with hypothyroidism secondary to AT will have an elevated TSH ("typically" over 10 IU/mL), a low FT_4, and positive anti-TPO antibodies. In early stages of the disease, TSH may be normal and anti-TPO antibodies may be positive with or without goiter. Later, TSH elevation becomes modest (5–10 IU/mL) with a normal FT_4 (biochemical or subclinical hypothyroidism). Up to 90% of patients with hypothyroidism secondary to AT are anti-TPO antibody positive. It should be noted that 10–15% of the general population are positive for anti-TPO antibodies and that low titers (less than 1/100 by agglutination methods or less than 100 IU/L by immunoassays) are less specific for ATDs [1].

If anti-TPO antibodies are absent, less common etiologies of primary hypothyroidism should be considered: transient hypothyroidism due to postsubacute thyroiditis, hypothyroidism related to external irradiation [49], and consumptive hypothyroidism due to the inactivation of thyroid hormone by the paraneoplastic expression of type 3 iodothyronine deiodinase, mostly in vascular tumors [50]. Subclinical hypothyroidism is defined as TSH elevation with normal concentrations of circulating thyroid hormones (FT_4 and FT_3). The log-linear relationship between serum TSH and FT_4 explains how small reductions in serum FT_4 lead to large deviations in TSH. The majority of these patients are asymptomatic, but studies in the adult population suggest that individuals with the combined risk factors of TSH level above the normal limit and positive thyroid antibodies (anti-TG or anti-TPO) are at high risk for progression to overt hypothyroidism. For this reason, we recommend thyroid hormone replacement in all patients with TSH values >10 IU/mL or with TSH values >5 IU/mL in combination with goiter or thyroid autoantibodies [51].

2.5. Therapy and Management. Levothyroxine (L-T_4) is the replacement therapy of choice. There are virtually no adverse reactions; its good intestinal absorption and its long half-life of 5–7 days allow oral administration once a day. Although very rare, the development of pseudotumor cerebri associated with the initiation of L-T_4 has been described in a small number of school-age children [52]. Some authors advocate a graded approach to the initiation of L-T_4 [53]. Alternatively, a starting dose can be estimated based upon the patient's age and ideal body weight (Table 2) [34]. The medication's long half-life insures a gradual equilibration over the course of 5–6 weeks, and dosing should be individualized on the basis of biochemical monitoring [34]. TSH normalization is the goal of replacement. In our practice, we aim to reach values in the lower part of the normal range (0.5–2 micro IU/mL). This will usually be associated with an FT_4 in the upper half of the normal range. Thyroid function tests should be obtained about 6–8 weeks after the initiation or subsequent adjustment of the L-T_4 dosage. Very high TSH levels at diagnosis can be associated with thyrotroph hypertrophy and gradual suppression over the first year of treatment [54, 55]. Growth and sexual development should be followed systematically as in any paediatric patient. Once biochemical euthyroidism has been achieved, TSH can be monitored every 4–6 months in the growing child and yearly up to the attainment of final height. If poor compliance is suspected as the cause of treatment failure, FT_4 should be measured. A serum TSH greater than twice normal, with a concomitant normal FT_4 level, suggests intermittent omission of the medication. A variety of conditions or drugs may alter L-T_4 requirements (Table 3). L-T_4 should be administered at least 20 min before eating or ingestion of any medication known to impair its absorption, such as calcium and iron supplements, sucralfate, potassium-binding resins, antacids containing aluminium, and bile-acids binding resins. All other medications should be checked for interactions, particularly with antidepressants and seizure medications (Table 3). Parents of children with AT should be advised that the hypothyroidism is likely to be permanent and monitoring of thyroid function for all patients should be lifelong. The prognosis for recovering lost linear growth depends on the duration of the hypothyroidism as well as the age at which treatment is started. If hypothyroidism is longstanding, thyroid replacement will not recover all lost stature. Similarly, if the diagnosis is made around puberty, there may be limited time for recovering the growth spurt before attaining final height. If the onset of childhood hypothyroidism occurs after age 2 to 3 years, no permanent intellectual damage or neurologic deficit is probable. Children affected by type 1 diabetes, celiac disease, and Down, Turner, and Williams syndrome should undergo annual thyroid function tests to ensure that hypothyroidism has not become evident.

Table 2: Recommended levothyroxine (L-T_4) treatment doses.

Age	Dose (mcg/kg/day)
0–3 months	10–12
3–6 months	8–10
6–12 months	6–8
1–3 years	4–6
3–10 years	3–4
10–15 years	2–4
>15 years	2–3
Adult	1.6–1.8

2.6. Natural History and Prognosis. The natural history of AT in children and adolescents is not fully known. Few studies have examined the spontaneous evolution of the disease [56, 57]. A recent Italian retrospective study described the outcome of 160 children affected with AT followed for up to 32.6 years in 20 paediatric endocrine clinics [58]. In agreement with other reports [56, 57], TSH concentrations showed large fluctuations over time. Analyzing all the data together, a trend toward progressively deteriorating thyroid function was evident. However, at the last observation 84

Table 3: Conditions that increase L-T_4 requirements.

Pregnancy
Mucosal diseases of the small bowel
Jejuno-ileal bypass and small bowel resection
Drugs which impair L-T_4 absorption (cholestyramine, sucralfate, aluminum hydroxide, calcium carbonate, and ferrous sulphate)
Drugs which may enhance CYP3A4 and thereby accelerate L-T_4 clearance (carbamazepine, rifampin, phenytoin, estrogen, and sertraline)
Drugs which impair T_4 to T_3 conversion (amiodarone)
Conditions which may block type 1 deiodinase (selenium deficiency due to dietary deficiencies as in phenylketonuria and cystic fibrosis)
Cirrhosis

patients (52.5%) still had a normal thyroid function or had become euthyroid. The authors analyzed reliable prognostic factors to predict disease evolution but found that clinical measures, thyroid volume, and antibody concentrations were similar in the group with normal TSH levels and the group with elevated TSH levels. The number of patients with type 1 diabetes was higher in the group with normal TSH. This can be explained by the fact that patients with autoimmune diseases are usually periodically assessed for thyroid autoimmunity, and thus many patients with mild asymptomatic forms of AT can be identified. Altogether, the presence of associated diseases did not worsen the prognosis, because at the end of the follow-up there was no difference in the frequency of abnormally elevated TSH between the groups with or without associated diseases. In agreement with previous findings in children [59–61] and in contrast with adults [62], the TSH level at baseline was not a useful marker to predict disease evolution. Both thyroid antibodies were significantly higher at the last visit in the group with deteriorating thyroid function; however, whereas anti-TG antibodies were already higher at baseline, anti-TPO antibodies increased progressively with time. This finding suggests that anti-TPO antibodies might represent a marker of deteriorating thyroid function, in agreement with a previous report showing a good correlation between anti-TPO antibodies levels and lymphocytic infiltration of the gland [63]. The evaluation of patients, according to their final outcome, revealed that subjects with deteriorating thyroid function had significantly higher anti-TG antibodies, TSH concentrations, and greater thyroid volume at presentation. Nonetheless, these findings were not helpful in individual patients. On the other hand, it should be remarked that at 5 years of followup, more than 50% of the patients remained or became euthyroid.

We usually offer a trial off L-T_4 therapy to adolescents, after the completion of growth and puberty. Thyroid function is retested 6–8 weeks after the stop of medication, to determine if hypothyroidism is permanent and potentially restart therapy.

2.7. Thyroid Nodules and Cancer in Patients with AT. Although unusual in children and adolescents, thyroid nodules are more often malignant in children than in adults [64, 65]. The prevalence of thyroid cancer among patients with AT is a matter of controversy. After Dailey et al. [66] reported 35 cases of AT among 288 patients with malignant thyroid disease, postulating that the disorder could be considered a precancerous lesion, other researchers seek an association between AT and thyroid cancer. Subsequent studies [67–69] reported a prevalence range of 1% to 30%. Data on the occurrence of thyroid cancer in AT refer almost exclusively to adults. The overall incidence of thyroid cancer among childhood thyroid nodules was estimated to be 26.4% in a review by Niedziela [69]. A recent study [64] analyzed the relationship between AT, cancer, and thyroid nodules in a large case series of paediatric patients. Thyroid nodules were found in 115 of 365 patients with AT (31.5%): 69 subjects (60.0%) presented a solitary nodule, and 46 subjects (40.0%) had multiple nodules. Thirty eight nodules (33%) were palpable at clinical examination, and the presence of all of them was confirmed by ultrasonography. Eleven cases of papillary carcinoma were detected on histologic examination after total thyroidectomy, with 5 exhibiting lymph node metastasis. Eight patients had multifocal cancer, and 3 patients had single focus cancer. The prevalence of male sex among patients with cancer was greater than that among patients with AT (odd ratio: 2.95). The finding of lymphadenopathy and the progressive increase of nodule diameter during L-T_4 therapy represented the two factors that were significantly more frequent in patients with thyroid cancer than in patients with a benign lesion. Thyroid ultrasonography provided further useful diagnostic information. Among patients with thyroid cancer, hypoechogenicity seemed to predominate over other ultrasound patterns, although it was common also in benign nodules. Multinodularity was more frequent than uninodularity in patients with cancer.

2.8. The Link between GD and AT. The observation that the autoimmune attack against the thyroid gland could result in two opposing clinical phenotypes, AT and GD, has been intriguing for decades.

In AT, the lymphocytic infiltration of the thyroid gland leads to apoptosis of thyroid cells and hypothyroidism. In contrast, in GD the lymphocytic infiltration of the thyroid leads to activation of TSHr-reactive B cells that secrete TSHr-stimulating antibodies causing hyperthyroidism. The etiology of AT and GD involves common pathways in which thyroid reactive T cells escape tolerance and infiltrate the thyroid, and unique pathways in which these thyroid-reactive T cells either cause thyroid cell death (in AT) or stimulation (in GD). Although GD and AT have different clinical phenotypes and the mechanisms leading to their dichotomy are unknown, they are generally believed to share a number of common etiological factors. There have been reports on monozygotic twins in whom one twin had GD and the other one had AT [70, 71]. Moreover, both conditions may aggregate in the same family [72] or may even coexist in the same thyroid gland [73], and some individuals may

progress from one form to the other. It is more frequent that GD may spontaneously culminate in hypothyroidism due to AT [74], while the development of GD from AT as only occasionally been reported [75, 76]. On the other hand, whole-genome scanning studies in humans have revealed differences between the specific loci linked to, or associated with, these two ATDs [77]. A recent study, performed in 109 children with GD at clinical onset [78], demonstrated that hyperthyroidism might be preceded by AT presenting either hypothyroidism or euthyroidism in at least 4 cases (3.7%). After AT diagnosis, 3 of these patients underwent L-T_4 therapy, which was continued for at least 1 year. In all these cases, a subsequent thyroid function evaluation, performed 1–3 months prior to the GD diagnosis, had evidenced normal FT_4 and TSH serum levels. The time interval between AT diagnosis and GD presentation ranged from 1.5 to 2.8 years. All of them exhibited both thyroid enlargement and other clinical signs as well as symptoms of hyperthyroidism, while exophthalmos and even mild eye signs were not evident in any of them. Serum TSHr antibodies were higher in the patients with no AT antecedents. The clinical course of GD in patients with AT antecedents was not different from the one observed in those with no AT antecedents.

Table 4: Clinical signs and symptoms of hyperthyroidism in children.

Goiter
Exophthalmos
Acceleration of linear growth
Irritability
Impaired concentration and school performance
Headache
Hyperactivity
Fatigue
Palpitations
Tachycardia
Systolic Hypertension
Polyphagia
Increased frequency of bowel movements with diarrhoea
Weight loss
Heat intolerance
Increased perspiration
Tremor
Polyuria and polydipsia

3. Graves' Disease (GD)

Robert Graves reported the clinical syndrome of goiter, palpitations, and exophthalmos in 1835. In adults, GD accounts for 60–80% of all patients with hyperthyroidism. Hyperthyroidism is relatively rare in children (yearly incidence of 8 per 1,000,000 children less than 15 years old and 1 per 1,000,000 children less than 4 years old), but GD is by far the most common etiology. Girls are affected four to five times more frequently than boys, although no gender difference is noted under 4 years of age [79].

3.1. Pathophysiology. GD shares many characteristics with AT, including anti-TG antibodies, anti-TPO antibodies, and antibodies against the sodium-iodine symporter. Hyperthyroidism is caused by thyroid-stimulating antibodies that bind and activate TSHr, leading to follicular cell hyperplasia and hypersecretion of thyroid hormones. Lymphocytic infiltration of the thyroid is present. Sometimes, germinal centers appear and develop as major sources of intrathyroid autoantibodies. The lymphocytic infiltration and the accumulation of glycosaminoglycans in the orbital connective tissue and skin cause the extrathyroidal manifestations of GD ophthalmopathy and dermopathy, respectively.

3.2. Clinical Aspects. The presentation of GD in childhood may be insidious and a careful history often reveals a several month history of progressive symptoms. Children may have the same signs and symptoms of hyperthyroidism as do adults, but most often they present with behavioral disturbances: decreased attention span, difficulty concentrating (which may lead to deteriorating performance in school), emotional lability, hyperactivity, difficulty sleeping, and nervousness. Typical cardiovascular findings include tachycardia, palpitations, widened pulse pressure, and an overactive precordium. Any child who has persistent tachycardia should be evaluated for hyperthyroidism. Tremors, a shortened deep tendon reflex relaxation phase, fatigue, and proximal muscle weakness are possible neuromuscular manifestations of thyrotoxicosis. Despite an increase in appetite, affected children often lose weight and sometimes have diarrhoea, but usually have frequent bowel movements associated with intestinal motility (Table 4). Increased perspiration, warmth, and heat intolerance tend to be late findings. Postpubertal girls often have menstrual irregularities. A goiter is palpable in the majority of cases, characterized by diffuse enlargement which is smooth, firm, and nontender. The pretibial myxedema that is a common feature of GD in adults is rare in children.

Extrathyroidal manifestations such as ophthalmopathy and dermopathy are rarer in children than in adults and tend to be less severe [80]. A 25–60% frequency of ocular manifestations has been estimated in children, but usually the ocular signs are mild such as lid retraction, a slight proptosis that can be attributed to the inflammation and muscle swelling rather than to infiltrative disease of the orbital structures. As expected, these signs improve in most patients after restoration of the euthyroid state [80]. Unique to pediatric GD is the acceleration of linear growth and bone maturation associated with prolonged hyperthyroidism [81, 82].

3.3. Diagnosis. Even if there may be national differences in terminology, for the purposes of this study the term thyrotoxicosis refers to the manifestations of excessive quantities of circulating thyroid hormones. On the contrary, hyperthyroidism refers only to the group of diseases which are due to the overproduction of hormones by the thyroid gland. An accurate diagnosis of GD is critical as antithyroid

drugs have no role in the treatment of thyrotoxicosis without hyperthyroidism. Thyrotoxicosis is recognized by an elevation of serum FT_4 with a decreased serum TSH (typically <0.1 micro IU/mL). A determination of the FT_3 level should be added if TSH is suppressed and the serum FT_4 is normal. In patients with early disease or in iodine-deficient patients, serum FT_4 concentrations may be normal or reduced despite elevated levels of FT_3. Once biochemical derangement has been documented, it is helpful to address the duration of thyrotoxicosis to facilitate the differentiation of GD from other causes of thyrotoxicosis. Onset may be documented by prior laboratory studies or inferred from the history. The differential diagnosis of thyrotoxicosis includes transient thyroiditis, hyperfunctioning nodules, and thyrotoxicosis factitia. In the majority of cases, the presence of a symmetrically enlarged thyroid gland, coupled with the chronicity of symptoms, will be adequate to allow a diagnosis (Table 5). If thyrotoxicosis has been present for more than 8 weeks, GD is by far the most likely etiology. The constellation of thyrotoxicosis, goiter, and orbitopathy is pathognomonic of this condition, and no additional laboratory tests or imaging studies should be necessary to confirm the diagnosis. If thyromegaly is subtle and eye changes are absent, a thyroid echography should be performed. The radioactive iodide uptake (RAIU) should be reserved for patients in whom a discrete nodule(s) is palpable or evident at ultrasonography. In patients with a toxic nodule, iodide uptake will localize to the nodule and the signal in the surrounding tissue will be low, secondary to TSH suppression. Thyrotoxicosis factitia can be recognized by a low RAIU and serum TG, in the presence of thyrotoxicosis and suppressed TSH levels. If thyrotoxicosis has been present for less than 8 weeks, transient thyrotoxicosis secondary to subacute thyroiditis or the thyrotoxic phase of AT should be considered. An elevated sedimentation rate supports subacute thyroiditis whereas increased TPO and TG without increased TSHr antibody titers supports the latter. RAIU was used in the past decades to distinguish thyrotoxicosis due to the different forms of thyroiditis (increased release of thyroid hormone—low RAIU), from the more common GD (increased production of thyroid hormone—high RAIU), but the measurement of TSHr antibodies may now offer an effective tool to make the correct diagnosis, and RAIU is no more indicated for differential diagnosis. Anti-TSHr antibodies are commonly present in GD, whereas they are absent from AT and in the other forms of thyrotoxicosis. The sensitivity of two frequently used serum anti-TSHr antibody assays is cited to be 75–96% for TBII (a competitive binding assay with TSH) and 85–100% for TSAb measurements (a bioassay of TSH receptor activation) in untreated GD patients. A false negative rate of 10–20% has been documented for serum anti-TSHr antibodies in GD, presumably due to the inadequate sensitivity of the assays, or the exclusive intrathyroidal production of autoantibodies.

In practice, the measurement of anti-TSHr antibodies is routinely used in children to avoid RAIU, as the combination of clinical signs, symptoms of thyrotoxicosis, and positive autoantibodies, in the absence of a nodule at ultrasonography, is virtually diagnostic of GD. There is a subgroup of patients who have a subnormal but not severely depressed TSH (usually 0.1–0.3 micro IU/mL) and normal serum concentrations of thyroid hormones. These patients are generally asymptomatic and the term "subclinical hyperthyroidism" has been applied to their condition. In elderly people, a low serum TSH concentration has been associated with an increased risk of atrial fibrillation, but no similar risks have been identified in the paediatric population [83]. Furthermore, several studies indicate that approximately half of patients with subclinical thyrotoxicosis will experience a spontaneous remission [84]. The initial detection of a suppressed TSH concentration, without elevated levels of thyroid hormone or associated symptoms, should be addressed simply by repeating thyroid function tests in 4–8 weeks. Assuming there are no specific risk factors such as a history of cardiac disease, asymptomatic children with subclinical hyperthyroidism can be followed with the expectation that TSH suppression due to transient thyroiditis will resolve spontaneously and that due to GD or autonomous secretion will declare itself clinically over time.

Table 5: Differential diagnosis of thyrotoxicosis in children.

Thyrotoxicosis associated with sustained hormone overproduction (hyperthyroidism). High RAIU
Graves' disease
Toxic multinodular goiter
Toxic adenoma
Increased TSH secretion (TSH secreting adenomas)
Thyrotoxicosis without associated hyperthyroidism. Low RAIU
Thyrotoxicosis factitia
Subacute thyroiditis
Chronic autoimmune thyroiditis
Ectopic thyroid tissue (struma ovarii, functioning metastasis of differentiated thyroid cancer)

3.4. Antithyroid Medications. The treatment of hyperthyroidism due to GD may be divided into two categories, antithyroid medications and definitive therapy. The thionamide derivatives, methimazole (MMI) and propylthiouracil (PTU), are the most commonly used antithyroid drugs [85]. Both thionamides block thyroid hormone biosynthesis and PTU, when used at doses over 450–600 mg per day, have the additional action of inhibiting the extrathyroidal conversion of T_4 to T_3. The recommended starting dose is 0.5–1.0 mg/kg per day for MMI and 5–10 mg/kg per day for PTU. Both drugs cross the placenta, although PTU does so less and is preferred during pregnancy. Although both are present in human milk, their concentrations are low, and breastfeeding may be continued. Due to its longer half-life, MMI can be administered once or twice a day, whereas PTU should be administered three times a day. Over the 60 years that this medication has been used, reports of PTU-related liver failure and death have been accumulated. The risk of severe PTU-induced liver failure is estimated as 1 in 2000–4000 children. The number of children developing reversible PTU-induced liver injury is estimated to be at

least 1 in 200 children. Routine biochemical surveillance of liver function and hepatocellular integrity is not useful in identifying children who will develop liver failure. Children appear to be at higher risk for PTU-induced liver injury than adults. PTU should not be used as first line therapy for the treatment of GD in children. Current PTU use in children should be stopped in favor of alternate therapies [86, 87].

For the specific situations of severe hyperthyroidism or thyroid storm, PTU has been the preferred thionamide because of its blockade of T_4 to T_3 conversion, through the inhibition of type 1 iodothyronine deiodinase. In such patients, a combination of high doses of PTU (up to 1200 mg per day divided in 4 doses) and inorganic iodine (SSKI: three drops orally twice a day, for 5–10 days) will speed the fall in circulating thyroid hormones. Some authors have advocated a "block and replace" strategy of high-dose antithyroid medication (to suppress all endogenous thyroxine secretion) combined with levothyroxine replacement. One report described a lower frequency of recurrence with this approach [88]. However, all subsequent studies have failed to duplicate this finding [89]. For the purpose of simplifying therapy and minimizing the risk of adverse effects, we usually prefer monotherapy with a single antithyroid medication. After FT_4 level has fallen to the upper end of normal range, the dose of antithyroid drug should be decreased by one half or one third. Further dose adjustments are guided by serial thyroid function tests, initially relying upon the FT_4. After pituitary TSH secretion recovers from suppression, the goal of maintenance therapy is TSH normalization. The "block and replace" approach is more complicated, but it can be useful in small children when the titration and tapering of the thionamide dose can be difficult: even very small doses are able to cause hypothyroidism, but the therapy cannot be stopped too early, due to the elevated risk of recurrence. The first clinical response to medications is usually evident after 2–4 weeks. Weight loss stops or weight gain occurs. Beta-adrenergic antagonists may be used as an adjunct during this interval but, as the cardiovascular manifestations of hyperthyroidism are generally well tolerated in children, we reserve this therapy for symptomatically significant palpitations. Antithyroid drugs are usually well tolerated, but side effects are seen more commonly in children than in adults. Thirty-six serious complications and 2 deaths in children have been reported to the FDA [90]. Agranulocytosis (defined as a granulocyte count less than 500/microL) is a serious idiosyncratic reaction that can occur with either MMI or PTU. For this reason, a baseline white count should be obtained prior to the initiation of antithyroid drugs, since mild neutropenia may be present in the GD patients prior to the initiation of treatment, and it will be repeated 7–10 days after the start of therapy. Families should be advised that fever, sore throat, or other serious infections may be manifestations of agranulocytosis and therefore should prompt the immediate cessation of antithyroid drugs, the notification of the physician, and a determination of white blood cell count.

Reports of long-term remission rates in children are variable, ranging anywhere from 30–60% [91]. Remission rates are considerably less in prepubertal (17%) compared to pubertal (30%) children, but a recent retrospective study of 76 paediatric patients describes a 38% rate of long-term remission achieved with more prolonged courses of antithyroid medication (mean treatment duration of 3.3 yr) [92]. If the dose of antithyroid medication required to maintain euthyroidism is 5 mg per day of MMI (or less for younger children) for 6 months to 1 year and the serum TSH concentration is normal, a trial of medication may be offered. Antithyroid drugs can be discontinued and TSH concentrations monitored at monthly intervals. If hyperthyroidism relapses, as indicated by suppressed TSH levels with elevated FT_4 levels, antithyroid medications should be resumed or definitive therapy provided.

3.5. Definitive Therapy. The two options for the definitive treatment of GD are I-131 and thyroidectomy. Both usually result in life-long hypothyroidism, and there is no agreement in the literature as to their indications. Some centers consider these modalities as options for the initial treatment of paediatric hyperthyroidism [93–95]. However, considering that a remission of GD occurs in a significant percentage of children, at the onset of the disease we recommend a long-term trial with antithyroid medications (at least 2 years of continuous therapy). If the disease relapses after stopping therapy, one of the definitive therapeutical options should be considered. If patient noncompliance prevents the successful treatment, or both antithyroid medications must be discontinued secondary to serious drug reactions, the choice of a definitive therapy is appropriate. Thyroid ablation by I-131 is the first choice definitive treatment in adults, but concerns over the potential long-term complications of paediatric radiation exposure have made endocrinologists cautious in applying this approach to children [96]. The literature about GD in adults describes an increased relative risk for the development of stomach cancer (1.3 fold) and breast cancer (1.9 fold), but no large, long-term, follow-up studies of patients treated under 16 years of age have appeared [97]. It is estimated that more than 1000 children have received I-131 for the treatment of GD. To date, there are no reports of an increase in the incidence of thyroid carcinoma or leukaemia in this population [96, 97]. Despite the reassurances of these reports, experience with X-rays and the Chernobyl nuclear power plant accident indicate that the carcinogenic effects of radiation to the thyroid are the highest in young children [98, 99]. This argues for continued surveillance and, for children who fail antithyroid medication, the provision of an I-131 dose adequate to destroy all thyroid follicular cells. Some institutions administer an empiric dose of 3–15 millicuries, or a dose based upon the estimated weight of the gland (50–200 micro CI per gram of thyroid tissue) [95–97]. Efficacy is dependent upon both thyroid uptake and mass, and it is more useful to prescribe a dose which will provide approximately 200 micro Ci/g estimated weight in the gland at 24 hours. Antithyroid drugs should be discontinued for 3 days prior to the administration of I-131. For children who are unable to swallow a capsule, a liquid preparation of I-131 is available

$$\text{Dose} = \frac{200 \text{ micro Ci/g} \times \text{g(thy)} \times 100}{\%\text{ uptake in } 24\,\text{h}}. \quad (1)$$

The frequency of acute side-effects is low although vomiting has been frequently described in paediatric patients [95]. One prospective study of 443 patients ranging from 15 to 85 years of age has raised the concern that I-131 may worsen or precipitate the development of Graves' ophthalmopathy in approximately 15% of cases [100]. Severe ophthalmopathy is less common in paediatric GD, but a study addressing the risk of this presentation in children is not available. The current paediatric literature suggests that the rate of ophthalmologic exacerbation is similar amongst the various treatment modalities: 3% after I-131, 2% with thionamide derivatives, and 9% after subtotal thyroidectomy [88]. A short course of glucocorticoids is appropriate if there is rapid progression of ophthalmopathy or as prophylaxis prior to radioiodine in children with pre-existing severe ophthalmopathy. Baseline opthalmological assessment at the onset of treatment is advisable.

Thyroidectomy is rarely used electively for the definitive therapy of GD, except with massive thyromegaly (over eight times normal size or thyroid weight >80 g) or for patients in whom coexisting nodules are suspicious for carcinoma by fine needle aspiration. A meta-analysis of the paediatric literature provided the following analysis of surgical treatment: subtotal thyroidectomy relieved hyperthyroidism in 80% of patients, with 60% becoming hypothyroid. Total thyroidectomy cured hyperthyroidism in over 97% of patients with nearly universal hypothyroidism. The overall complication rate in children included a 2% incidence of permanent hypoparathyroidism, a 2% incidence of vocal cord paralysis, and a 0.08% mortality [90]. One large institution has published a series of 82 children treated surgically over 14 yr with much better results. Bilateral subtotal resection was the most frequently performed operation (86%) and, with a median followup of 8.3 yr. A recurrence rate of 6% is reported, while no cases of permanent recurrent laryngeal nerve palsy, permanent parathyroid disease, or death were observed [101]. The difference between the average complication rate and those in a single institution emphasizes the importance of skill and experience in the performance of this procedure [102]. Postoperative hypothyroidism is expected and it is easily treated, and all GD patients require life-long monitoring. We suggest to consider thyroidectomy only for patients who have persistently failed medical management or those whose parents or physicians do not wish to proceed with radioiodine therapy. Based on the results to date, I-131 therapy is an acceptable alternative if the surgical options are undesirable. I-131 is recommended for all patients who recur following surgery, due to the high complication rate of secondary thyroidectomy [103].

3.6. Neonatal GD. Thyroid hormones are necessary for optimal foetal and neonatal development, and the risk of malformations may be increased in the newborns to hyperthyroid mothers [104, 105]. Lack of thyroid hormones for more than a few weeks, during vulnerable periods of development, involves a risk of permanent cerebral impairment [106]. Conversely, excess amounts of thyroid hormone are associated with increased risk of foetal death and may lead to accelerated bone maturation leading to early epiphyseal fusion and growth cessation. Also long-term exposure may lead to ostepenia in adolescence and adulthood [107]. Only 0.6% of infants born to mothers with a history of GD will develop neonatal hyperthyroidism, due to the transplacental passage of thyroid-stimulating immunoglobulins. Even after definitive treatment by I-131 or thyroidectomy, women with a history of ATDs are at risk for foetal and neonatal thyroid dysfunction secondary to the persistence of maternal autoantibodies. The pregnancy of such women should be considered high risk, and the care should be coordinated between an experienced obstetrician and an endocrinologist. Foetal heart rate and growth should be monitored by regular prenatal ultrasounds. The measurement of anti-TSHr antibodies during at-risk pregnancies has been recommended as a predictor for the development of foetal/neonatal GD [108]. Highly experienced ultrasonographers can often visualize the foetal thyroid. The presence of foetal goiter, tachycardia, and intrauterine growth retardation suggests foetal hyperthyroidism. In these rare patients, antithyroid drugs are administered to the mother to control foetal hyperthyroidism, this will keep the foetus euthyroid until birth. After birth, the antithyroid drugs from the mother will disappear from the foetal circulation within the first days of life. After some delay, neonatal hyperthyroidism may develop and remain until the maternal antibodies are cleared. Paediatricians should be aware that the use of maternal antithyroid medications near the time of delivery or the co-transfer of maternal anti-TSHr blocking immunoglobulins may delay the appearance of neonatal GD [107]. For high-risk infants, such as those born to mothers with high levels of anti-TSHr stimulating antibodies or those with a history of an affected sibling, clinical monitoring and thyroid function tests at birth and at 1 and 2 months of age are recommended [109]. An additional set of laboratory tests at 1 week of age is indicated for infants who have been exposed to maternal antithyroid drugs in the third trimester. Affected infants are often flushed, diaphoretic, and hyperkinetic. Goiter is common and, when severe, can endanger the infant's airway. Diarrhoea, vomiting, poor weight gain, and a transient exophthalmos may be seen. Arrhythmias and/or congestive heart failure can develop and require treatment with digoxin. Serum for confirmatory thyroid function tests (TSH, FT_4) should be obtained and treatment initiated immediately. PTU (5–10 mg/kg per day) or MMI (0.5–1.0 mg/kg per day) may be administered, orally or by gastric tube, in divided doses every 8 hours. MMI is preferred following reports of serious PTU toxicity. Inorganic iodine will speed the fall in circulating thyroid hormone, using saturated solution of potassium iodide (SSKI) (48 mg iodide/drop) at the dose of one drop per day. Iopanoic acid or sodium ipoiodate have also been used for their iodine content and their capacity to inhibit the activation of T_4 to T_3. As in older patients, adjunctive therapy with beta-blockers (propranolol: 2 mg/kg per day—in 4 doses) and glucocorticoids (prednisone: 2 mg/kg per day—in 2 doses) may be helpful in severe cases. Cardiac failure may occur in some cases and require treatment with digoxin. During the period of foetal and neonatal hyperthyroidism, the pituitary TSH secretion has been suppressed and the phase of neonatal hyperthyroidism

may be followed by a phase of secondary hypothyroidism, until pituitary TSH secretion is restored.

The cumulative morbidity of neonatal Graves was estimated to be as high as 25% in the past, although it appears to be considerably lower today [108, 109]. Potential long-term morbidity includes growth retardation, craniosynostosis, impaired intellectual function, and central hypothyroidism. The half-life of maternal immunoglobulin is approximately 14 days, so most cases of neonatal Graves will resolve after 3–12 weeks (depending upon the initial levels of anti-TSHr antibodies). The history of maternal illness is critical. Adolescent women who have GD should know that, even if they are "cured," when they become pregnant, their babies eventually will be at risk.

The differential diagnosis of neonatal thyrotoxicosis includes the McCune-Albright syndrome, activating mutations of the TSH receptor and thyroid hormone resistance syndrome [109, 110]. These non-autoimmune etiologies are rare but should be considered if thyrotoxicosis persists beyond 3 months of age.

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Research Article

Invariant NKT Cell Lines Derived from the NOD·H2^{h4} Mouse Enhance Autoimmune Thyroiditis

Rajni B. Sharma,[1] Xiaoguang Fan,[1] Patrizio Caturegli,[1] Noel R. Rose,[1,2] and C. Lynne Burek[1,2]

[1] *Department of Pathology, School of Medicine, Bloomberg School of Public Health, Johns Hopkins Medical Institutions, Johns Hopkins University, Ross Research Building, 648, 720 Rutland Avenue, Baltimore, MD 21205, USA*
[2] *Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins Medical Institutions, Johns Hopkins University, Ross Research Building, 648, 720 Rutland Avenue, Baltimore, MD 21205, USA*

Correspondence should be addressed to C. Lynne Burek, lburek@jhmi.edu

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To study the role of invariant Natural Killer T cell (iNKT) cells in autoimmune thyroiditis, we derived two iNKT cell lines from the spleens of NOD· H2^{h4} mice, a strain that develops spontaneous autoimmune thyroiditis exacerbated by excess dietary iodine. The two lines were CD1d-restricted and expressed CD4$^+$, DX5$^+$, and the Vα4Jα281 gene segment, of the T-cell receptor α locus. Upon stimulation with α-galactosyl-ceramide (α-GalCer), both lines rapidly produced IL-2, IL-4, IFN-γ, IL-10, and TNF-α. Strikingly, a similar cytokine response was also induced by thyroglobulin, one of the most abundant protein in the thyroid gland and a major autoantigen in human autoimmune thyroiditis. Transfer of the iNKT cell lines to syngeneic hosts enhanced autoimmune thyroiditis. Intraperitoneal injections of α-GalCer in iodine primed mice also induced thyroid disease. This paper reports for the first time that iNKT cells respond to thyroglobulin and enhance autoimmune thyroiditis in iodine fed NOD·H2^{h4} mice.

1. Introduction

Autoimmune thyroiditis, also known as Hashimoto's thyroiditis, ranks third in prevalence among the autoimmune disorders in the United States [1], and is determined both by genetic and environmental factors [2]. Some aspects of Hashimoto's thyroiditis can be duplicated using the NOD·H2^{h4} mouse, a strain that spontaneously develops thyroiditis at a low incidence. The incidence and severity of thyroiditis can be exacerbated by supplementation of sodium iodine (NaI) in the drinking water; almost 100% of NOD·H2^{h4} mice drinking NaI-enriched water for 6 to 8 weeks develop moderate to severe thyroiditis [3, 4]. The immunopathology of NOD·H2^{h4} thyroiditis is similar to that of Hashimoto's thyroiditis and is characterized by the development of autoantibodies to thyroglobulin and chronic infiltration of the thyroid gland by mononuclear cells such as CD4$^+$ T cells, CD8$^+$ T cells, B cells, and macrophages [4–6].

Little is known about the role of regulatory immune cells in the pathogenesis of autoimmune thyroiditis. Recent studies have shown that the iNKT cells are a unique population of lymphocytes that downregulate several autoimmune diseases, such as type 1 diabetes and experimental autoimmune encephalomyelitis [7, 8]. Other contrasting studies, however, have shown that iNKT cells may be pathogenic as shown in CD8 T cell-induced NOD diabetes [9] and Con A-induced hepatitis [10, 11]. Thus, since both disease improvement and exacerbation have been shown, even within the same disease model (NOD type I diabetes), the decisive role of iNKT cells in autoimmune pathogenesis remains unclear.

Characteristically iNKT cells share receptors of both T cells and NK cells (NK1.1 and/or DX5) [9, 12] and have an alpha chain of their T-cell receptor encoded by invariant gene segments, Vα14 Jα18 in mice, and Vα24-JQ in humans [13–15]. Two novel antigenic targets for iNKT cells, an exogenous microbial cell antigen and an endogenous lysosomal

glycosphingolipid isoglobotrihexosylceramide (iGb3), have been discovered [16, 17]. Most iNKT cells promptly respond to a synthetic ligand α-galactosyl-ceramide (α-GalCer) and secrete a variety of cytokines characteristic of both Th1 (IFN-γ) and Th2 type (IL-4 and IL-13) responses [18, 19]. iNKT cells interact with target hydrophobic antigens in the context of CD1d, a nonpolymorphic, MHC class I-like molecule usually expressed on conventional antigen presenting cells (APC) [13, 20]. Several studies have documented extensive heterogeneity and diversity in iNKT cell populations that has led to their classification into several subsets [18, 19]. A unique subset of iNKT cells has also been documented that bears CD1d on their surface, lack NK1.1, and autopresent α-GalCer in the absence of conventional APC [21].

Previous studies have shown that adoptive transfer of thyroglobulin-stimulated splenocytes induced autoimmune thyroiditis in mice [22, 23]. However, the precise nature of the specific immune cells that are responsible for transferring disease has not been well delineated. In this paper, we describe two lines of iNKT cells that express surface phenotype of $CD4^+DX5^+$, are CD1d-restricted, autopresent thyroglobulin, and produce both Th1 and Th2 cytokines. We report that these lines of iNKT cells are nonprotective and enhance thyroid autoimmunity in iodine-fed NOD·$H2^{h4}$ mice.

2. Materials and Methods

2.1. Mice. NOD·$H2^{h4}$ mice were bred and maintained in the Johns Hopkins University conventional animal facility. Both male and female mice aged 10 to 12 weeks were used in the studies. Test mice were fed 0.15% of NaI in their drinking water and control mice received regular tap water.

2.2. Purification of Mouse Thyroglobulin. Thyroglobulin was purified as previously described [24]. Briefly, mouse thyroid glands were dissected and homogenized in protease inhibitor buffer. Debris was removed by centrifugation and the supernatant was applied to a 1.6×88 cm Sephacryl S300 column (Sigma-Aldrich chemicals, St. Louis, MO) equilibrated with PBS at 4°C. The protein content of each column fraction was determined by spectrophotometry (OD_{280}) and finally by BCA protein assay kit (OD_{560}) (PIERCE, Rockford, IL, USA). Small aliquots of thyroglobulin were collected and frozen at −20°C until used.

2.3. Endotoxin Test. During the thyroglobulin purification process, precautions were taken to avoid any microbial or endotoxin contamination. All thyroglobulin preparations were analyzed using quantitative chromogenic QCL-1000 LAL-test kit bought from Bio-Whittaker, Walkersville, MD, USA. Assay was performed according to the manufacturer's protocol. Briefly, a series of twofold dilutions of endotoxin standards (0.5 EU/mL to 0.008 EU/mL) was prepared. Pyrogen-free, endotoxin-tested water (EU < 0.03, Invitrogen Corporation, Carlsbad California) was used to prepare samples and as a negative control. Serial dilutions of 50 μL of either test or standard samples were prepared and incubated for 10 minutes at 37°C with 100 μL of Limulus amoebolysate, and then with 100 μL of chromogenic substrate for 6 minutes. The reaction was stopped using 100 μL of Stop solution. The absorbance was read spectrophotometrically at 405 nm on ELISA plate reader (Dynatech Laboratories, USA). Thyroglobulin preparations with <0.125 EU were considered endotoxin-free and were used in all assays.

2.4. Thyroglobulin Antibody-Specific ELISA. Purified thyroglobulin was coated onto 96 well Immunolon II plates (Dynatech, USA) at a concentration of 2 μg/mL in carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed 4 times with PBS-Tween 20 (0.05%) and blocked for 2 hrs with 1% BSA-PBS. Plates were then washed 3 times and incubated overnight with mouse sera diluted 1 : 100 in PBS. Mouse thyroglobulin-specific IgG subclasses were detected using appropriate dilutions of secondary antibodies against IgG1 and IgG2b (ICN Inc., Aurora, OH). Color was developed with *p*-nitrophenylphosphate substrate (Sigma, St. Louis, MO). Optical density (OD_{405}) was read on MRX plate reader (Dynatech Laboratories, USA).

2.5. Cell Preparation and Development of Cell Lines. NOD·$H2^{h4}$ mice were fed a low dose of sodium iodide (0.05%) in drinking water for 12 weeks. Spleen mononuclear cells (MNCs) were isolated by density gradient centrifugation on Ficoll Paque (Pharmacia, Biotech, Sweden). Cells were washed and cultured in 24 well plates at a density of 10^6 cells/mL with complete RPMI 1640 supplemented with 10% FBS, 20 IU/mL penicillin, 20 μg/mL streptomycin, 20 mM/L L-glutamine, and 100 μM nonessential amino acids (all from GIBCO BRL, MD, USA). Bone marrow derived dendritic cells were used as feeders. IL-2 (10 ng/mL), IL-4 (100 U/mL), and GM-CSF (50 ng/mL) (PharMingen, San Diego, CA) were added to the cells every 3-4 days, and the cells were stimulated with thyroglobulin every 14–18 days. The cells proliferating in response to thyroglobulin were selected for emergent cell lines and were then developed in 96 well plates at a density of 3×10^{-2} cells/mL. Two lines 1F1 and 2D11 were selected; line 1F1 was derived twice and named as 1F1.1. Bone marrow derived dendritic cells from the same mouse strain were used as feeder layers. Control cell line was derived from an MHC II matched OVA transgenic mouse. $CD4^+$ cells were isolated by magnetic separation using CD4 (L3T4) microbeads (Miltenyi Biotech Inc. Auburn, California USA). Cells were cultured in 6 well plates in supplemented complete medium RPMI 1640 and pulsed with 5 μM chicken $OVA_{323–339}$peptide (ISQAVHAAHAEINEAGR) (Fort Collins, CO USA) using adherent APCs for 24 and 48 hours before adoptive transfer.

2.6. Antibodies and Flow Cytometry. Surface expression of iNKT cells was analyzed using fluorescein isothiocyanate (FITC)/peridinin chlorophyll protein (PerCP) conjugated anti-CD4 (L3T4) and phycoerythrin-(PE-) coupled pan

NK anti-DX5 monoclonal antibodies (mAb). PE-coupled mCD1d mAb (clone 1B1) (BD PharMingen, San Diego, CA, USA) was used to analyze CD1d expression. Intracellular cytokine expression was measured using monoclonal antibodies to mouse IL-2, IL-4, IFN-γ, IL-10, and TNF-α coupled to PE (PharMingen, San Diego, CA). Three-color staining was used to analyze cell surface markers, and intracellular cytokines using standard protocol provided by the manufacturer (BD PharMingen, San Diego, CA, USA). Briefly, developed iNKT cells were stimulated for 4 hours with 45 μg/mL of thyroglobulin in complete RPMI-1640 and incubated for last 2 hours with Golgi stop (BD PharMingen, San Diego, CA, USA). The cells were washed and stained for 30 minutes with cell surface markers followed by cell fixation and staining with intracellular cytokine markers. Flow cytometric analysis was done on a FACSCalibur with Cellquest software (Becton Dickinson, Heidelberg, Germany).

2.7. Expression of Vα14Jα281 in iNKT Cells. Mouse iNKT cells express a TCR$\alpha\beta$ that utilizes invariantly the Vα14 and Jα281 gene segments [15, 25]. Total RNA was extracted from each cell line (RNA easy Mini kit, QIAGEN, Valencia, CA) and reverse transcribed using the primer 5′-TGGCGTTGGTCTCTTT-GAAG-3′, which binds to the constant α region of the TCR. PCR amplification was then performed using the upstream primers 5′-TCCTGGTTGACCAAAAAGAC-3′, which binds to the V α14 region. The 443 bp amplicon was separated on a 2% agarose gel.

2.8. Proliferation Assay. A three-day proliferation assay was performed to test the response of the iNKT cell lines to thyroglobulin. Cells (2×10^4 cells/well) were cultured for 72 hours in complete RPMI-1640 along with adherent peritoneal macrophages as APCs and were stimulated with 45 μg/mL of thyroglobulin. To assess whether the thyroglobulin-specific proliferation of iNKT cells depends on CD1d engagement, proliferation assays were performed with different concentrations of purified mouse anti-CD1d mAb for blocking (3.0–0.19 μg/well, 0 representing no mAb). Cells were pulsed with 1 μCi of [methyl-3H] thymidine (Amersham, Pharmacia, NJ, USA) for the last 18 hours. Proliferation was measured as incorporated [methyl-^{3}H] thymidine on a matrix 96 direct β-scintillation counter (Wallace, Germany). Data represents mean values of triplicate wells after 4 minutes of counts on the beta-counter.

2.9. CD1d Tetramer Staining. Recombinant soluble CD1d protein (gift from Dr. A. Bendelac) was incubated with thyroglobulin (100 ng) or with a 2 μM solution of αGalCer in 0.005% Tween 20; unbound protein was removed by centrifugation dialysis in a Microcon YM-30 tube (Millipore, Bedford, MA). Tetramers were formed by mixing thyroglobulin or αGalCer-loaded monomers with PE-conjugated streptavidin (at 5 : 1). Staining was done by incubating cells on ice for 3 h with tetramers at a concentration equivalent to 3–5 μg/mL of CD1d1. For the IL-2 assay NKT hybridoma was similarly incubated with loaded CD1d and secretory IL-2 was measured in the supernatant.

2.10. Adoptive Transfer. iNKT cell lines were incubated with thyroglobulin for 4 hrs at 37°C in a humidified, 5% CO_2 incubator prior to transfer into 10-to-12 week-old NOD·H2^{h4} mice. The cells were washed twice and resuspended in sterile PBS and injected intravenously (i.v.) on days 0 and 2 at a concentration of 5×10^6 cells/mouse. All mice were fed 0.15% NaI in their drinking water 2 weeks prior to cell transfer. Mice were sacrificed 14 days postinjection; thyroids were dissected, and sera collected for detection of autoantibodies to thyroglobulin. Control groups received iodine water and injections of sterile PBS i.v. at same time as test mice instead of cell transfers. As a control cell line, OVA-specific CD4$^+$ cells were similarly transferred to iodine fed and age-matched mice after stimulation with 5 μM chicken OVA$_{323–339}$ peptide (ISQAVHAAHAEINEAGR) (Fort Collins, CO USA) using adherent APCs for 24 or 48 hours before adoptive transfer.

2.11. Tracking of Transferred Cells. In order to track these cells, iNKT cell lines which were derived from Thy1.2 NOD·H2^{h4} mice were adoptively transferred to iodine fed Thy1.1 NOD·H2^{h4} mice at concentrations described above. Cells were isolated from the thyroids after 14 days posttransfers and were analyzed after enzymatic digestion by flow cytometry as previously described [26].

2.12. In Vivo α-GalCer Treatment and Iodine Priming of Mice. NOD·H2^{h4} mice were used: experimental group $n = 9$ and control groups of Vehicle alone $n = 5$ and iodine alone group $n = 7$. Both males and females were included in the study. Iodine was supplemented in their drinking water for two weeks prior to α-GalCer injections. Two α-GalCer i.p. injections of 100 μg/mouse on days 0 & 7 were given. Mice were sacrificed on day 14.

2.13. Tissue and Serum Collection. Sera were collected prior to iodine water treatment (day-14), on the day of adoptive transfer of cells (day-0), and at day 14 posttransfer. Thyroids were fixed in 10% buffered formalin for 2 days, and submitted for histological staining. Blood samples were incubated at room temperature for 30 minutes; sera were collected after centrifugation and stored at −80°C until used.

2.14. Histology. Paraffin embedded sections of thyroids were stained with hematoxylin and eosin and graded from 0 to 4 score based on the extent of mononuclear cell infiltration. A grade of 0 was assigned for no lesions, 1 for <20% infiltration, 2 for 20–30%, 3 for >30–50%, 4 for >50% infiltration of the thyroid.

2.15. Statistics. All comparisons of normally distributed data were made using Student's t-test; otherwise, Mann-Whitney

U test was used. Test values were considered to be statistically significant from control values at $P < .05$.

3. Results

3.1. Adoptive Transfer of Cell Lines Resulted in Autoimmune Thyroiditis. Two iNKT cell lines were derived from the spleen cells of NOD·H2^{h4} mice stimulated with thyroglobulin as described in methods. The possible role of these cell lines in autoimmune thyroiditis was first determined. Adoptive transfers were performed with both iNKT cell lines along with appropriate control cells such as OVA-specific CD4+ cells. Adoptive transfer experiments with both cell lines were performed in iodine pretreated NOD·H2^{h4} mice. Mice were sacrificed at day 14 following adoptive transfer, and results were analyzed by (i) scoring thyroid histopathology, and (ii) assessing thyroglobulin antibody by ELISA.

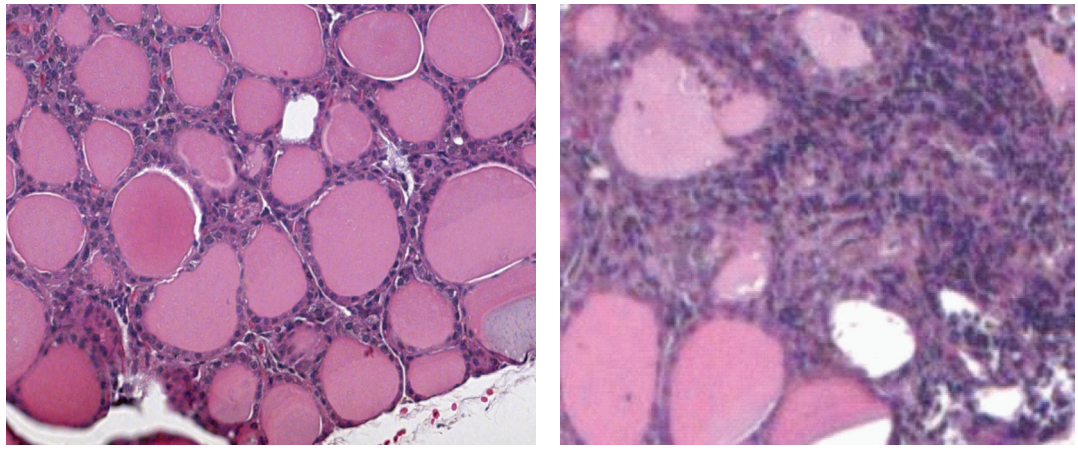
3.1.1. Thyroid Histology Showed Increased Cellular Infiltration. Histological analysis of the cellular infiltrates of mice receiving either cell line 1F1.1 or 2D11 cells revealed moderate to dense cellular infiltration scoring from 2-3 (30–50%) as well as intense follicular destruction as compared to controls (Figures 1(a) and 1(b)). Table 1 shows a summary of results of disease frequency and severity of lesions developed postadoptive transfer. Two control groups were used; one group received iodine but no cell transfer and other did not receive iodine but did receive equivalent number of cells as the experimental groups. The control group that received NaI in their drinking water for same time period as the experimental group did not develop lesions in the thyroid except for one mouse that developed a low level of thyroiditis, probably due to the spontaneous phenotype of the mouse model. The adoptive transfer of line 1F1.1 resulted in development of lesion scores from 1–3 in 8 of 12 mice. Similarly line 2D11 resulted in lesion score of 1-2 in all 4 of 4 mice (Table 1). Adoptive transfer of control OVA-specific CD4$^+$ cells showed no infiltration of the thyroid glands in any of the mice (Table 1).

3.1.2. Thyroglobulin Antibody Levels Increased after Adoptive Transfer of NKT Cells. Thyroglobulin-specific IgG1 and IgG2b autoantibody subclasses were detected in the serum of iNKT cell transfer recipients. Figure 2 shows results from a representative adoptive transfer experiment from line 1F1.1. Significantly increased levels of IgG1 (Figures 2(a) and 2(b)) ($P < .005$) and IgG2b antibodies (Figures 2(c) and 2(d)) ($P = .02$) to thyroglobulin were seen in almost all of the mice receiving transfers in comparison to control mice that received NaI alone (Figure 2). Since the production of autoantibodies to thyroglobulin is indicative of thyroid autoimmunity, these results suggested that all of the mice receiving 1F1.1 cells in this particular experiment ($n = 9$) developed enhanced response to thyroid autoantigens culminating in thyroiditis. None of the mice that received control OVA-specific CD4$^+$ cells developed antibody to thyroglobulin (data not shown). Since we now knew that our cell lines could induce autoimmune thyroiditis in NaI-treated NOD·H2^{h4} mice, we proceeded to characterize these cells in detail.

3.2. Proliferative Response of Cell Lines to Mouse Thyroglobulin. To show that the cell lines respond to thyroglobulin, we performed an *in vitro* proliferation assay. The two cell lines, 1F1.1 and 2D11, were cultured for 72 hours at a cell concentration of 2×10^4/well on irradiated adherent peritoneal macrophages with 45 μg/mL of thyroglobulin. The same cell lines were cultured with either ovalbumin or medium alone as controls. Both cell lines showed a significantly higher proliferation in response to thyroglobulin ($P = 7.9499\text{E} - 05$); however, both lines also showed a weak response to ovalbumin (Figure 3). Thus, we hypothesized that iNKT cells that are strongly responsive to our thyroglobulin preparation enhance thyroid autoimmunity and contribute to disease.

3.3. Intracellular Cytokine Profiles of Cell Lines. iNKT cells promptly produce various cytokines in response to α-GalCer. To determine the intracytoplasmic cytokine expression, the two lines were stimulated with thyroglobulin or α-GalCer. Intracellular immunofluorescent staining was performed at various time points (2, 4, 8, 18, 24, and 48 hours), to determine the optimal time-point for the intracellular expression of cytokines from the two lines (data not shown). After performing the kinetics, we found that both lines expressed intracellular cytokines within 4 hours of stimulation. The expression of most cytokines disappeared after 10 hours. Therefore, the 4-hour time point was used for further assays and analyses. Interestingly, both cell lines displayed different patterns of intracellular cytokine production (Figures 4(a) and 4(b)). Although both cell lines had high numbers of IL-2-producing cells initially, but upon long-term culture they lost this ability of IL-2 secretion. Line 1F1.1 showed a polarization towards Th1 response. High number of IFN-γ producing cells (approximately 72–82% with thyroglobulin or α-GalCer resp.) and few IL-4 secreting cells (~2%) were recorded as shown in Figure 4(a). Line 2D11 showed moderate numbers of both, IFN-γ (approximately 50–54% with thyroglobulin or α-GalCer resp.) and IL-4 (approximately 28–44% with thyroglobulin or α-GalCer respectively) producing cells (Figure 4(b)). Thus, line 2D11 showed a different cytokine profile as compared to line 1F1.1. IL-10 was found in significant proportions of cells in both the lines with 25–70% in 1F1.1 (with thyroglobulin or α-GalCer resp.) and 38–42% in line 2D11 (with thyroglobulin or α-GalCer, resp.). TNF-α was found in almost all the cells of line 1F1.1 but only 30–35% cells of line 2D11. Thus, although the variation in the numbers of cytokines secreting cells existed among the two lines, the pattern of cytokines within the lines in response to either thyroglobulin or α-GalCer was similar. Furthermore, upon repeated stimulation, the percentages of cytokine producing cells of a particular line remained constant (Figure 4 represents data from one representative experiment).

3.4. Characterization of the Phenotype of Cell Lines. iNKT cell lines that proliferated in response to thyroglobulin and



(a) (b)

Figure 1: A representative figure of thyroid gland histology from a control mouse and a adaptively transferred with NKT cell line 1F1.1 is shown after hematoxylin and eosin (H & E) staining. (a) Normal thyroid histology showing follicles surrounded with thyrocytes. (b) Section from thyroid gland after 14 days after adoptive transfer of iNKT cells line 1F1.1. Cellular infiltration and disruption of normal thyroid histology was observed. The thyroid histology was assessed as a 5-point score as described in Table 1.

Table 1: Incidence and severity of thyroiditis after transfer of iNKT cell clones to NOD·H2^{h4} mice.

2-week administration of NaI	Transferred iNKT cell clone	Thyroiditis incidence	Thyroiditis severity*				
			0	1	2	3	4
Yes	—	1/12	11	1	0	0	0
No	1F1.1	0/4	4	0	0	0	0
Yes	1F1.1	8/12	4	3	4	1	0
Yes	2D11	4/4	0	1	3	0	0
Yes	CD4$^+$ (OVA specific)	0/4	4	0	0	0	0
Yes	*α-GalCer*	5/9	4	1	3	0	1
Yes	*Vehicle*	1/7	6	1	0	0	0

* Thyroiditis severity was scored as follows: 0 for no lesions, 1 for <20% infiltration, 2 for 20–30%, 3 for >30–50%, and 4 for >50% infiltration of the thyroid.

produced both Th1 and Th2 type cytokines were characterized for the expression of various cell phenotypic markers. The cells were stained for the characteristic surface markers associated with T cells (TCR$\alpha\beta$, CD4, and CD3) and NK cells (DX5, a pan-NK cell marker). Unstimulated cell lines were also stained to determine the constitutive expression of various surface markers. Both the cell lines in resting as well as stimulated states expressed surface markers for TCR$\alpha\beta^+$, CD4$^+$, CD3$^+$, DX5$^+$, and CD69$^+$ as shown in Table 2. In addition to common iNKT cell markers, our iNKT cells also expressed CD1d on their surface. In order to detect whether macrophages could be present in the cell cultures, accounting for CD1d expression, macrophage/dendritic cell markers (Mac1, CD80, and CD86) were also tested. None of the cell lines showed detectable levels of such markers for macrophages, dendritic cells, or other populations such as CD8$^+$ or B220$^+$. However, coexpression of CD4$^+$ and DX5$^+$ was detected on 95–99% cells of both cell lines (Table 2, Figure 5). It is not surprising that NK1.1, a common marker for NK cells, was not observed on the cell lines since NK cells from parental NOD mice do not display this allelic marker [12].

Although these results suggested that the lines are indeed a subset of iNKT cells, further confirmation was required. A classical characteristic of most iNKT cells is their restricted usage of the invariant chain TCR$\alpha\beta$ encoded by Vα14Jα281 gene rearrangement [14, 15]. Importantly, both cell lines expressed Vα14Jα281 as shown by RT-PCR (Figure 6). In contrast, a CD4$^+$ NK1.1$^-$/DX5$^-$ T cell clone, used as a negative control, did not show any such expression (Figure 6). These results confirm that the cell lines produced and stimulated by our thyroglobulin preparation derived from NOD·H2^{h4} mice are a subset of iNKT cells. Hence, the mixed Th1/Th2 cytokine profile from these cells as shown above is not surprising, since autoimmune thyroiditis shows both Th1 and Th2 cytokines with disease pathology [5, 27], and iNKT cells are also known to release large amounts of both types of cytokines following four hours of stimulation.

3.5. CD1d-Restriction of iNKT Cell Lines. Most iNKT cell subsets recognize lipids or hydrophobic peptide antigens in the context of CD1d, that is usually expressed on antigen presenting cells [28]. We found that our iNKT cell lines

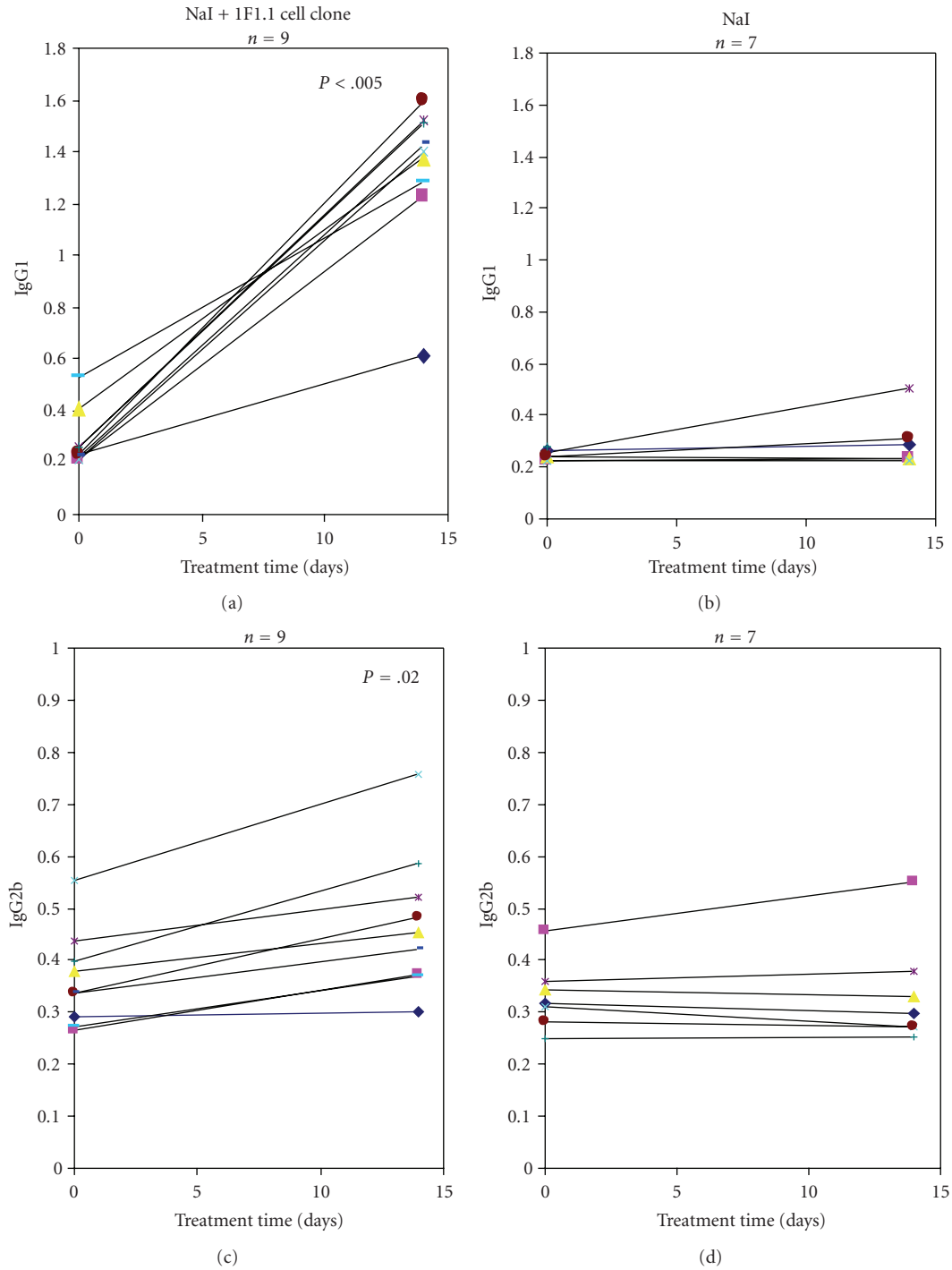


Figure 2: Adoptive transfer of iNKT line 1F1.1 in 8–10-week-old syngeneic mice induced antibodies to thyroglobulin. Mice in panels (a) and (c) received pretreatment of iodine and received iNKT cells. Both, IgG1 and IgG2b (a and c) antibody titers in the treatment groups (posttransfer day 14) were significantly higher as compared to the control group of mice (b and d). Shown IgG1 with $P < .005$ and IgG2b with $P = .02$. Control group mice on panels (b) and (d) received iodine pretreatment but no cells. No significant increase in the antibody titer to thyroglobulin was seen in control groups.

expressed CD1d and proliferated in response to thyroglobulin in the absence of conventional APCs (Figure 7). The proliferation assay results suggested that CD1d bearing iNKT cells are capable of auto-presenting antigen in absence of conventional APCs as described earlier by Hameg et al. [21].

To test the dependence of the cell lines on CD1d for stimulation by thyroglobulin, we blocked CD1d using a CD1d monoclonal antibody (mAb). We found that thyroglobulin-specific proliferation was completely abrogated in a dose-dependent fashion with CD1d mAb treatment, whereas

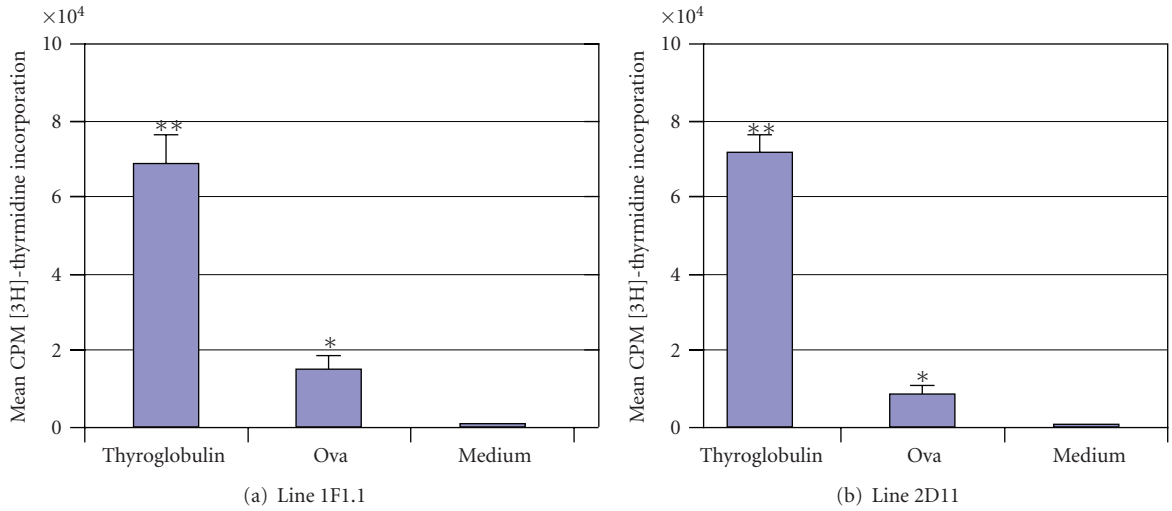


Figure 3: *In vitro* response of iNKT cells to thyroglobulin. A 72-hour proliferation assay was performed in response to 45 μg/mL of thyroglobulin. [3H]-thymidine incorporation was used as an indicator of stimulation response. Both cell lines showed a significantly higher proliferation in response to thyroglobulin. A few cells also proliferated in response to Ovalbumin (Ova). Results are expressed as mean counts/4 minutes of [3H]-thyrmidine uptake. (Representative of 3 independent experiments).

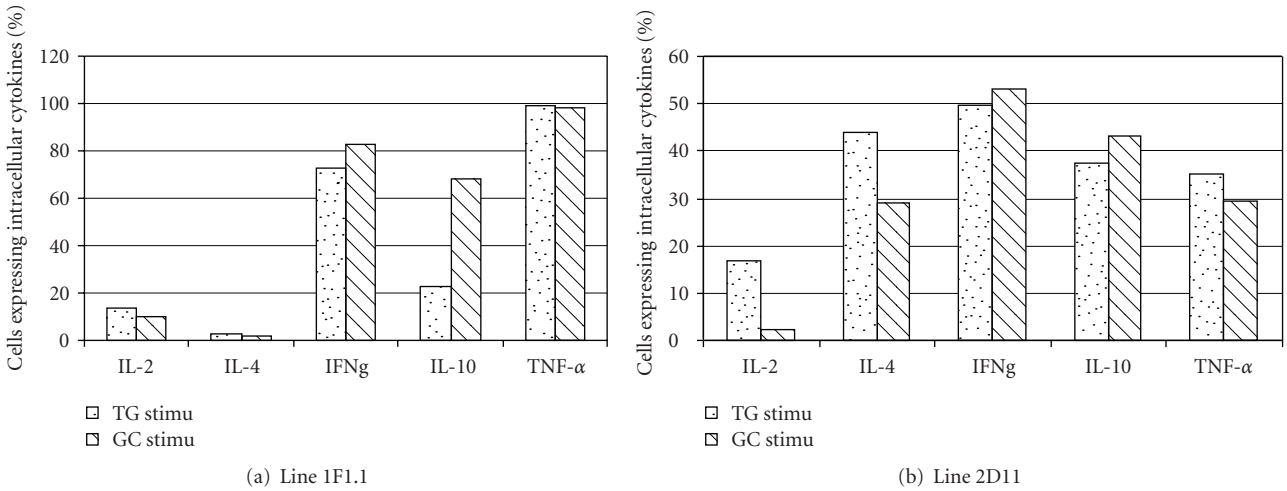


Figure 4: Cytokine response of two iNKT cell lines. Cells were stimulated with thyroglobulin or α-GalCer four hours prior to staining. Intracellular cytokine expressions were then detected by flow cytometry as described in methods. Both lines showed a burst of cytokines after 4 hours of stimulation. (a) Line 1F1.1 showing high levels IFN-γ, but low levels of IL-4. (b) Line 2D11 showing elevated levels of both IFN-γ and IL-4. Data represents percent of total iNKT cells expressing intracellular cytokines.

unblocked cells efficiently proliferated in response to thyroglobulin stimulation (Figure 7).

To confirm the CD1d specificity of iNKT cell lines, we used tetramers for CD1d. FACS staining using α-GalCer-CD1d-specific tetramers confirmed that the lines are iNKT cells. Clones cells gated on $CD4^+DX5^+$ were found to be >88% positive for CD1d tetramer staining (Figures 8(a) and 8(b)). These results verify that our cell lines are functionally CD1d-restricted and recognized α-GalCer like typical iNKT cell clones.

3.6. In Vivo Treatment of α-GalCer Enhanced Thyroiditis in NOD·H2^{h4} Mice. Mice were given two α-GalCer injections i.p. after a short period (two weeks) of iodine treatment. As shown in Table 1, 55% of mice that received α-GalCer injections developed infiltration of the thyroid gland after 14 days. Approximately 22% of mice (2 of 9) also developed autoantibody to thyroglobulin (data not shown). From the control group, only 14% of mice (1 of 7) developed low-grade thyroid histology, but none of them developed detectable levels of thyroid autoantibody (Table 1).

3.7. Tracking iNKT Cells in the Thyroids. Since iNKT cells contribute to autoimmune thyroid autoimmunity in transfer experiments, it is important to determine the site of action of their adoptive transfer. To address this question

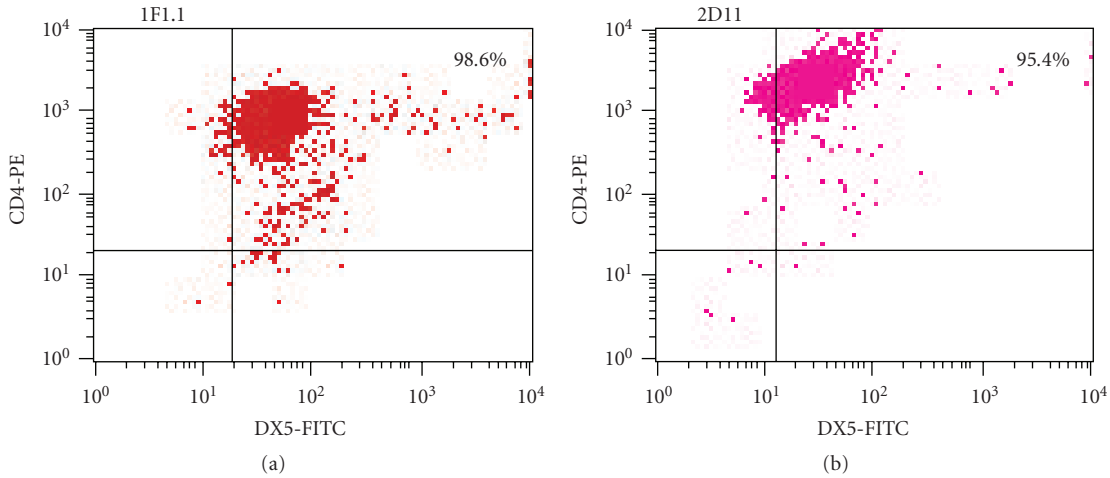


Figure 5: Surface phenotypic expression of two iNKT cell lines by two-color flow cytometry. Cells stained with mouse monoclonal antibody CD4-PE and DX5-FITC were analyzed after a side scatter versus forward scatter gate on live lymphocytes. Both iNKT clones showed a double-positive expression $CD4^+DX5^+$ on their surface. Line 1F1.1 showed a pure population of >98% and line 2D11 >95%.

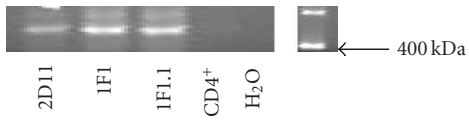


Figure 6: Expression of Vα14 Jα281 on iNKT cells. Mouse iNKT cells express a TCR$\alpha\beta$ that utilizes invariant Vα14 and Jα281 gene segments. RT-PCR using specific primers showed that lines 1F1, 1F1.1 subderived line of 1F1 and 2D11, all expressed Vα14Jα281 region, whereas a standard $CD4^+$ T cell clone was negative for this expression. The 443 bp amplicon was separated on a 2% agarose gel.

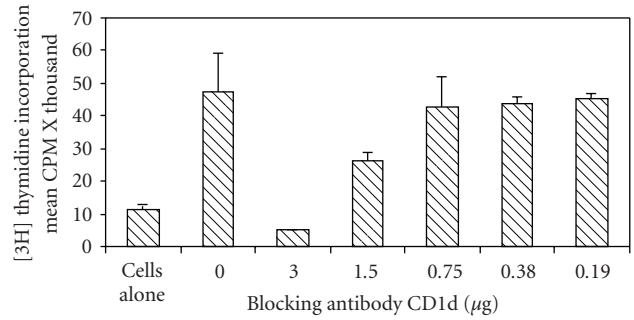


Figure 7: Demonstration of CD1d dependence of iNKT cells. Proliferation of line 1F1.1 in response to thyroglobulin was assessed after blocking CD1d. Different concentrations of mouse CD1d mAb were used starting from 0.0–3.0 μg/well. Concentration 0 represents no blocking. Proliferation in response to thyroglobulin was abrogated after CD1d blocking showing that iNKT cells recognized thyroglobulin in a CD1d-dependent manner. [3H] thymidine incorporation was measured as an indicator of proliferative response. Data represents mean CPM of triplicate wells from each concentration.

we performed adoptive transfer experiments using iNKT cell lines from $NOD{\cdot}H2^{h4}$ mice with Thy1.2 expression into $NOD{\cdot}H2^{h4}$ mice expressing Thy1.1. The cells were transferred in a similar manner as described earlier. Thyroids were collected for disease assessment 14 days following cell transfers. Single-cell suspensions were prepared and analyzed for detection of Thy1.2 iNKT cells. No infiltrating Thy1.2 expressing iNKT cells were detected by flow cytometry analysis (data not shown). However, we could detect $CD45^+$ infiltrating lymphocytes on day 14, indicating disease progression. We interpret these results to show that (i) these cells were short lived and/or (ii) influenced disease development indirectly through their cytokines most probably in the local lymph nodes but not intrusive in the thyroid gland itself.

4. Discussion

In this paper, we report that adoptive transfer of iNKT cell lines enhanced spontaneous thyroiditis in susceptible recipient mice. The characterization of the cell lines was established initially by detecting the surface phenotype of both $CD4^+$ and $DX5^+$ (a pan-NK cell marker), and then by the expression of invariant TCR α-chain: Vα14Jα281. Both cell lines were found to coexpress $CD4^+$ and $DX5^+$ on their surface. NK1.1, a marker commonly used to identify NK cells, was not detected on either of cell lines. This is not surprising since NOD mice, along with many other mouse strains do not express the NK1.1 antigen [12]. The expression of DX5 protein has been shown to be positive for all mouse strains studied and hence has been widely used as a marker to identify NK cells [9, 13]. The overall phenotypic analysis of the cell lines indicated that the vast majority of cells within the culture, at least 95–99% of the cells, expressed the iNKT cell phenotype.

In order to gain a better understanding of the functional characteristics of the iNKT cell lines, we examined their response to thyroglobulin or ovalbumin stimulation. In addition to proliferation in response to thyroglobulin, both lines responded weakly to ovalbumin. CD1d is known to have a much deeper groove than the classical MHC

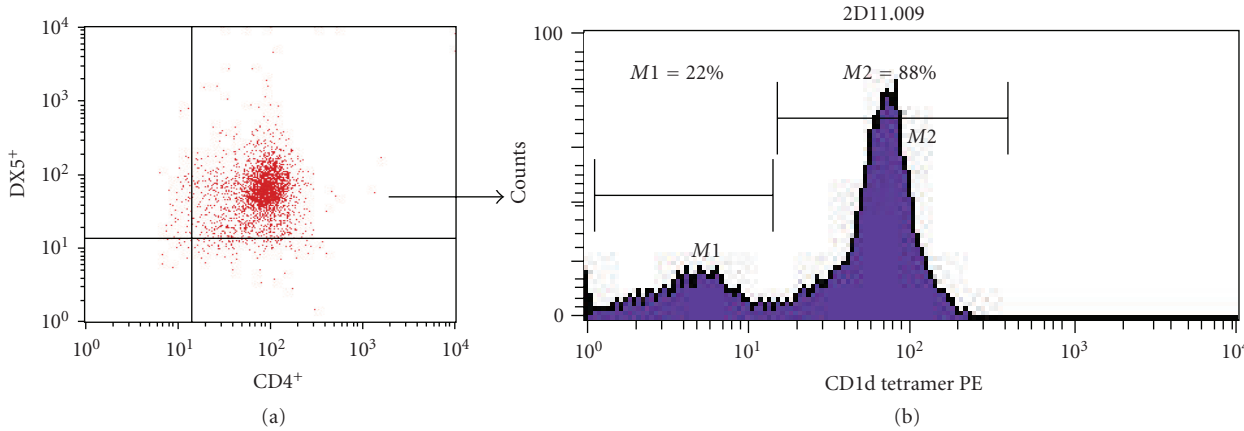


Figure 8: CD1d tetramer staining to show CD1d-restriction of iNKT cells. Flow cytometry was used to determine the percentage of tetramer positive cells from the homogeneous population of $CD4^+DX5^+$ iNKT cells. (a) Surface phenotype showing $CD4^+DX5^+$ of line 2D11. (b) Tetramer positive cells represented under M2 gate showing 88.8 % CD1d tetramer positive cells.

molecules, which binds with a high affinity to glycolipids and hydrophobic peptides [28]. It is known that the hydrophobic end of ovalbumin also binds to the CD1d groove with high affinity. Perhaps thyroglobulin, having many hydrophobic areas, is similarly presented by CD1d to iNKT cells [29]. Even though subsets of iNKT cells recognize antigens presented by CD1d, little is known about the role of exogenous hydrophobic peptide antigens, such as thyroglobulin or other natural ligands, in the processing, presentation, selection, and development of iNKT cells. Our proliferation data suggest that thyroglobulin, or a derived peptide, may be a candidate ligand for CD1d-dependent iNKT cell stimulation in iodine-fed NOD·$H2^{h4}$ mice. Iodine modification may still further contribute to the hydrophobic nature and stability of the thyroid autoantigen [30, 31].

The presence of CD1d on the surface of the iNKT cell lines suggests that these cells may be able to present thyroglobulin in an autocrine or paracrine manner. Because CD1d blocking inhibited the stimulation of these cells, it confirmed their CD1d restriction. However, it is not yet clear how thyroglobulin is processed. A study on characterization and sequence of human thyroglobulin (hTg) recognized the disease-inducing effect of a 40-amino acid (F40D) peptide from hTg. The pathogenic F40D peptide of human thyroglobulin was found to be highly hydrophobic in nature and located at the end of the second third of the thyroglobulin molecule. Injection of this peptide into thyroiditis-susceptible CBA/J mice strain induced severe autoimmune thyroiditis [32]. It is a possibility that iNKT cells may recognize hydrophobic peptide F40D or a similar hydrophobic peptide, leading to pathogenicity in NOD·$H2^{h4}$ similar to CBA/J. Alternatively iNKT cells may autopresent antigen by a mechanism similar to a subset of $CD8^+$ T cells that autopresents α-GalCer [21]. Recent studies demonstrate that human CD1d restricted T cells via $\alpha\beta$TCR, under certain inflammatory and autoimmune conditions, are capable of recognizing molecular structures of nonlipid small peptide molecules [33]. It appears that processing of antigen may take place by more than one way in vivo depending on the nature of antigen resulting in generation of pathogenic immune responses against more than one epitope of thyroglobulin. A recent study has shown that a plasminogen-like protein that is present in the apical region of thyroid epithelial cells naturally degrades thyroglobulin in order to maintain the concentration of thyroglobulin in the lumen of thyrocytes [34]. We speculate that during this process of degradation small hydrophobic antigenic fragments are formed that could be presented to iNKT cells in context of CD1d. Therefore, studying the factors promoting pathogenic epitopes during the processing and presentation of thyroglobulin by CD1d-bearing APCs should help to learn more about the recognition of thyroglobulin by iNKT cells and their role in disease pathogenesis.

The unique capacity of iNKT cells to promptly release cytokines upon antigenic stimulation is thought to be the basis of their regulatory functions during the effector phase of the immune response, especially in regulation of autoimmune disorders [8, 35]. iNKT cells may downregulate disease either by secreting cytokines that are protective [7] or by recruiting tolerogenic dendritic cells [36]. FACS analysis of the intracellular cytokine profiles of our iNKT cell lines revealed a diverse cytokine profile representing both Th1 and Th2 types after 4 hours of thyroglobulin stimulation (Figure 4) as supported by previous studies from iNKT cells stimulated *in vitro* [37, 38] or *in vivo* [39]. The diverse cytokine profiles of iNKT cells are known to be related to the nature of the antigen that stimulates them [38, 40]. For example, α-GalCer has been used to determine the functional significance of iNKT cell populations in the protection or prevention of autoimmune diseases such as type 1 diabetes and experimental autoimmune encephalomyelitis where protection of mice was associated with biased Th2 response [35, 40].

Stimulation of our iNKT cell lines with thyroglobulin or α-GalCer revealed only slightly different cytokine profiles between the two lines. After stimulation with either thyroglobulin or α-GalCer, both cell lines rapidly produced certain key cytokines such as IL-2, IL-4, IFN-γ, IL-10, and

Table 2: Surface phenotype of two lines of iNKT cells. Both lines expressed markers for T and NK cells.

Surface markers	1F1.1	2D11	CD4 (control)
T-cell markers			
TCR$\alpha\beta$	+	+	+
CD3	+	+	+
CD4	+	+	+
CD8	−	−	−
CD69	+	+	+
NK cell markers			
DX5	+	+	−
NK1.1	−	−	−
Ly6	+	+	−
APC markers			
CD1d	+	+	−
Mac1	−	−	−
CD80	−	−	−
CD86	−	−	−

TNF-α. Although the iNKT cell lines produced slightly different cytokine levels, both were capable of enhancing disease in genetically susceptible mice. Since iNKT cells were stimulated only with the thyroglobulin preparation, as presented by CD1d molecules, different epitopes of thyroglobulin could possibly be a ligand of iNKT cells. We thus suspect that in NOD·H2^{h4} mice and possibly in susceptible humans, autoimmune thyroiditis is enhanced indirectly by the cytokine products of iNKT cells in response to thyroglobulin stimulation.

Although Th1-type CD4$^+$ T cells are considered to be the predominant contributors to the initiation and persistence of autoimmune thyroiditis [5, 41], our study using thyroglobulin-stimulated iNKT cell lines that express IL-2, IFN-γ, IL-10, and TNF-α implicate a role for iNKT cells in the enhancement of autoimmune thyroiditis. A significant increase in serum levels of IgG1 and IgG2b antibodies to thyroglobulin 14 days after adoptive transfer of iNKT cell lines further suggests a key role of iNKT cells in the enhancement of thyroidal autoimmunity. The precise mechanisms involved in the enhancement of autoimmune thyroiditis by these iNKT cell lines are still unclear. Work by other investigators has clearly demonstrated that NKT cells promote autoimmune disease under certain conditions [10, 11, 42, 43]. It has been suggested that the stage of disease in which NKT cells are introduced into the experimental system play a major role in the outcome of disease. For example, if given early, prior to the start of disease the result is protection, but if given after initiation of disease, the result is disease enhancement [44]. In our experimental model of iodine-induced thyroiditis in the NOD·H2^{h4} mouse, transfer of the 1F1.1 iNKT cell line promoted disease only in iodine-primed mice, further suggesting that the role of these cells is enhancement rather than initiation of disease.

In summary, iNKT cell lines were derived from spleens of NOD·H2^{h4} mice by repeated stimulation with a mouse thyroglobulin preparation. These cell lines have all the molecular and functional earmarks of iNK T cells. When these lines were transferred into iodine-primed NOD·H2^{h4} recipients, thyroid autoimmunity was enhanced. In this model system of iodine-induced thyroiditis, these iNKT cells may be producing large quantities of Th1 cytokines, such as IFNg or TNFα, that dominate a more protective role of the Th2 cytokine response. These experiments lend further caution about NKT cell therapy for autoimmune diseases, as what may be protective in one could lead to disease enhancement in another.

Abbreviations

APC: Antigen-presenting cell
BM: Bone marrow
Ci: Curie
ELISA: Enzyme-linked immunosorbent assay
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate
GM-CSF: Granulocyte/macrophage colony-stimulating factor
h: Hour
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
i.p.: Intraperitoneal(ly)
i.v.: Intravenous(ly)
mAb: Monoclonal antibody
NK cell: Natural killer cell
OD: Optical density
OVA: Ovalbumin
PBS: Phosphate-buffered saline
RT: Reverse transcription
PCR: Polymerase chain reaction
TCR: T-cell receptor for antigen.

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Review Article

Challenges in Interpretation of Thyroid Function Tests in Pregnant Women with Autoimmune Thyroid Disease

Ulla Feldt-Rasmussen,[1] Anne-Sofie Bliddal Mortensen,[1] Åse Krogh Rasmussen,[1] Malene Boas,[2] Linda Hilsted,[3] and Katharina Main[2]

[1] *Department of Medical Endocrinology, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark*
[2] *Department of Growth and Reproduction, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark*
[3] *Department of Clinical Biochemistry, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark*

Correspondence should be addressed to Ulla Feldt-Rasmussen, ufeldt@rh.dk

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Physiological changes during gestation are important to be aware of in measurement and interpretation of thyroid function tests in women with autoimmune thyroid diseases. Thyroid autoimmune activity is decreasing in pregnancy. Measurement of serum TSH is the first-line screening variable for thyroid dysfunction also in pregnancy. However, using serum TSH for control of treatment of maternal thyroid autoimmunity infers a risk for compromised foetal development. Peripheral thyroid hormone values are highly different among laboratories, and there is a need for laboratory-specific gestational age-related reference ranges. Equally important, the intraindividual variability of the thyroid hormone measurements is much narrower than the interindividual variation (reflecting the reference interval). The best laboratory assessment of thyroid function is a free thyroid hormone estimate combined with TSH. Measurement of antithyroperoxidase and/or TSH receptor antibodies adds to the differential diagnosis of autoimmune and nonautoimmune thyroid diseases.

1. Introduction

Diagnosing maternal thyroid dysfunction during all stages of pregnancy is very important for the outcome for both mother and foetus [1, 2]. Women with hypothyroidism treated insufficiently with levothyroxine (high serum concentration of thyrotropin (TSH) or serum free thyroxine (T4) in the low normal range) deliver babies with significantly lower IQ and/or other inhibited neuropsychological development [3, 4]. Such offspring outcome has even been demonstrated in women with a serum concentration of T4 in the low normal range during pregnancy [5].

Prevalence of autoimmune thyroid disease (AITD) is high in women of reproductive age, whether or not they are pregnant [6]. AITD not only affects fertility [6], but may also lead to a decreased thyroid reserve with decreased availability of thyroxine. This is particularly important in the first half of pregnancy, in which the foetal development depends on the delivery of thyroxine from the mother [7, 8]. Although autoimmune thyrotoxicosis, Graves' disease, is rare in pregnant women, transfer of TSH receptor antibodies, which can be either stimulating or blocking, may give rise to foetal and neonatal thyrotoxicosis or hypothyroidism, respectively [9, 10].

As a natural consequence of the importance of thyroid hormones for foetal brain development much focus has been given to diagnosing both overt and subclinical (or mild) thyroid dysfunction as early as possible in pregnant women, recently resulting in international consensus guidelines [10]. Although the guidelines do not recommend universal screening of all pregnant women, most specialised clinical caretakers would attempt at including as many women as possible in a case finding programme. Women with autoimmune thyroid diseases or a family history of such belong to the risk groups [10].

Apart from general global problems in accomplishing this type of care due to financial and/or infrastructure restrictions, there are also many other reasons why these

efforts have limited success. One of them is associated with the biochemical measurements of thyroid function undergoing many complicated changes during pregnancy, and the corresponding issue of educating these important matters to the physicians who are caretakers of pregnant women. The question of whether precise detection and adequate treatment of thyroid insufficiency in pregnancy are feasible is still unanswered but recent progress and better insights into physiological changes, trimester-specific reference ranges, and intra- versus interindividual variability on the assessment of thyroid function in the single pregnant woman should give a better background for the future [11–13]. The present paper will focus on the choice of tests for assessment of biochemical thyroid function in pregnant women with AITD, together with their strengths and limitations. Information from two recent guidelines have been used in part as reference [10, 14] as well as the web-based textbook: www.thyroidmanager.org/ [15].

2. Physiological Changes during Pregnancy and Consequences for Thyroid Function Assessment

Normal pregnancy entails complicated and substantial changes in thyroid function [15]. The circulating thyroid hormone binding globulin (TBG) increases due to an oestrogen-induced increase in its production and at the same time the serum iodine decreases, the synthesis of thyroid hormones is increased, there are changes in the deiodinase activity, and, toward the end of the first trimester, when chorionic gonadotropin (HCG) levels are the highest, a significant fraction of the thyroid-stimulating activity is from HCG. Furthermore, thyroid autoimmune activity—reflected by thyroid autoantibody concentrations in serum—is usually decreasing due to a general immune suppressive action from the pregnancy, and finally plasma volume expands by approximately 50%, resulting in, for example, a lower serum albumin concentration. Serum concentrations of total T4 and T3 increase due to the increase of TBG. Serum concentrations of free thyroid hormones and TSH should physiologically be within normal limits, except in the short period of time when TSH may become suppressed due to the HCG effect (gestational hyperthyroidism). But it must be emphasised that normal reference ranges from a non-pregnant population are not to be considered "normal" in pregnancy. All the above-mentioned physiological changes, including the high TBG concentration, influence the laboratory measurements even of the free thyroid hormones. There is therefore a huge risk of false interpretation of thyroid function tests in pregnancy [16].

3. Biochemical Diagnosis of Thyroid Dysfunction in Pregnancy

3.1. Measurement of Serum TSH. The most sensitive method for screening for thyroid dysfunction in a healthy, non-pregnant population is the measurement of TSH serum concentration due to the log-linear relationship between TSH and free T4: even small changes in T4 concentrations will provoke very large changes in serum TSH. However, in pregnant women thyroid and pituitary functions are not stable, and, therefore, measuring TSH is not sufficient and often inappropriate for the assessment of thyroid function during gestation. If serum TSH measurement is used alone, the mother is likely to be insufficiently treated with levothyroxine for hypothyroidism or overtreated with antithyroid drugs for thyrotoxicosis, both of which resulting in maternal hypothyroidism, which in turn seriously affects the foetal brain development.

A typical example of such biochemical misdiagnosis during followup of antithyroid drug-treated Graves' disease is demonstrated in one of the cases from our tertiary referral department [17]. A 32-year-old 24-weeks pregnant woman was referred from a local hospital due to the finding of a large foetal goitre by routine scan. It was her second pregnancy, and she had been treated with antithyroid drugs for Graves' disease for 9 years. This included treatment during a previous pregnancy 5 years before, which resulted in a male baby with severely reduced cerebral capacity. Upon referral she was treated with 20 mg thiamazole daily. She had at the local hospital been considered sufficiently euthyroid based on a normal TSH of 2.9 mU/L (population-based reference range 0.4–4.0 mU/L), total T4 97 nmol/L (60–140 nmol/L), and free T4 7.8 pmol/L (7–20 pmol/L). She had a high level of TSH receptor antibodies at 24 U/L (<1.5 U/L). A more elaborate foetal ultrasound showed a male foetus with polyhydramnios and an enlarged thyroid gland with measures of $1.4 \times 3.5 \times 3.5$ cm, which was predominantly intrathoracic. Cord blood TSH was highly elevated at 34.5 mU/L, and free T4 was reduced to 13.8 pmol/L. The misdiagnosis of the thyroid function had been based primarily on the normal maternal levels particularly of TSH, which were, however, reflecting a delayed pituitary reaction to the slightly lowered free thyroid hormone levels. It is mandatory for doctors taking care of pregnant women with thyroid diseases to have a thorough knowledge of the evolution of the normal thyroid function during pregnancy as well as during treatment of thyroid dysfunction in order to avoid such unfortunate and unnecessary cases [17].

3.2. Measurement of Total or Free Thyroid Hormones. Measurement of the peripheral thyroid hormones themselves is complicated by a number of problems, the most important of which is the relationship to the gestation-induced elevation of the serum concentration of TBG. Since mostly immunoassays are used, biased values can derive from thyroid hormone antibodies in a woman with autoimmune thyroid disease, or heterophilic antibodies interfering in either the assays for TSH or thyroid hormones [18]. The elevated total hormone concentrations during gestation can display diverse reactions in the free thyroid hormone assays, either performing by giving a correct value or in most situations resulting in either over- or undercorrection. Consequently, results of free thyroid hormone measurements may very likely be either over- or underestimated leading to wrong diagnosis. A more reliable free thyroid hormone estimate is provided by measurement of total hormone concentrations (T3 and T4)

and correction for the increased binding proteins by either direct measurement of TBG (to provide T4/TBG or T3/TBG ratios) or a T3 or T4 uptake test. The latter can be calculated into free thyroid hormone indices, but as discussed in a very recent paper [19] these indices may also be incorrect during late pregnancy, probably due to insufficient correction of such extreme elevation of binding proteins, for which the methods are not designed. A qualitative or semiquantitative assessment of the total hormone concentrations and binding protein measurement separately may, however, be useful, and more so than the single free thyroid hormone results. It is important to note that the availability of these measurements depends on the local clinical biochemical laboratory.

From a clinical biochemical point of view total hormone measurements and creation of free thyroid hormone measurements are strictly the most reliable tests with the highest precision and accuracy. Very recently free thyroid hormones have been measured by equilibrium dialysis isotope dilution tandem mass spectrometry, which provides accurate, precise, fast, and simple measurements [20–24]. Such methods are, however, not generally available yet, and most laboratories still use immunoassays. In a recent paper, 3 immunoassays were compared with tandem mass spectrometry, and 2 of the 3 assays performed similarly to tandem mass spectrometry in late pregnancy, even when all 3 assays were dependant on binding proteins [19]. Thus, overall, the results of thyroid function testing during pregnancy are still puzzling and difficult and often even impossible to interpret.

4. Do Trimester-Dependent Reference Ranges Solve the Problem of Assessing Thyroid Dysfunction in Pregnancy?

Another problem in thyroid function tests is the population-based reference ranges, because they depend not only on the composition of the population and the iodine intake but also highly on the laboratory methods used. Therefore, there is a strong need of laboratory-dependent reference ranges, in order not to rely only on the reference range provided by the assay manufacturer. Because the progression of pregnancy and foetal, neonatal, and child health are dependent on adequate thyroid hormone supplementation throughout pregnancy, trimester-specific reference intervals for thyroid functions can be crucial for both maternal and foetal health. The physiologic changes associated with pregnancy require an increased availability of thyroid hormones by 40% to 100% to meet the needs of mother and foetus. Trimester-specific population-based reference ranges in order to correct for the physiological changes with increasing total hormone concentration and de- or increasing free hormones and suppressed TSH have been published from many sources in recent years [13, 24–37]. This approach will reduce the global variability of thyroid hormone assessment by approximately 6–18% [27], but it is important to emphasize that, for this to occur, the use of laboratory and population-specific ranges is crucial, since measurements by different methods in different populations do provide very different ranges (examples shown in Table 1). The table is not extensive but just examples of the most recent publications are shown. When producing trimester-specific reference ranges it is important that seemingly normal women with thyroperoxidase antibodies should not be included in the population [36].

5. The Problem of Population-Based Reference Ranges

Another, probably even more important, problem complicating the use of population-based reference ranges also in pregnant women is that each individual has its own genetic setpoint, as it has been shown by Feldt-Rasmussen et al. [12] and recently by Andersen et al. [38] in a nonpregnant population. In the initial studies when the methods for measurement of thyroid function had a lower sensitivity and higher imprecision the intraindividual coefficient of variation (CV%) was between 6 and 17% [12], also confirmed in a recent publication using more modern methods [39], while the interindividual CV% was 11 to 25%. A more relevant way of evaluating this is through an individuality assessment which was in these studies below 0.5, indicating that the thyroid function cannot be meaningfully assessed by the populations-based reference ranges [12, 13, 38–40]. Boas et al. [13] found a similar magnitude of variability in healthy pregnant women with an interindividual variability of 13–20% for both total and free T3 and T4, independent of gestational week, and an intraindividual variability of the same variables of 8–10%.

It is therefore very possible that also during pregnancy changes within the same woman are more important than the specific single measurement in relation to a specific reference range [13, 41]. In this case it applies also when using gestation-specific reference ranges, although the latter have to be considered also. This will result in a reduction of the total variability of the thyroid hormone function tests. The mentioned gestation-specific reference ranges should, however, be assessed in a given population with the given assays used in the laboratory and should not solely be based on information from the kit manufacturer, from the literature, or from another neighbouring laboratory, even if this laboratory uses the same assay.

6. Thyroid Autoantibodies

The measurement of thyroid autoantibodies in pregnant women is mainly useful to substantiate the probability of thyroid dysfunction in biochemically unclear cases, to ensure a correct differential diagnosis in case of maternal dysfunction, to predict the risk for development or deterioration of maternal thyroid dysfunction, and in few situations to predict for intrauterine and/or neonatal dysfunction [10, 14, 15]. Affection of the foetus and neonate is probably exclusively related to the presence and placental transfer of TSH receptor antibodies, which can also predict maternal hyperthyroidism in 60–70% of cases. When it comes to hypothyroidism the NHANES III study found almost similar prevalences of antithyroperoxidase and antithyroglobulin

Table 1: Trimester-specific reference ranges in various studies. Only a sample of studies is shown in order to exemplify the variety of values obtained in different populations of pregnant women and by different methods. Free thyroid hormone values are given—in some of the studies also total hormones have been measured together with T3 uptake to perform a free T4 index/estimate. Not all studies excluded pregnant women with thyroid autoantibodies.

			TSH			Free T4			Free T3		
		Method	1st trim	2nd trim	3rd trim	1st trim	2nd trim	3rd trim	1st trim	2nd trim	3rd trim
Boas et al. [13]	All trim Longitudinal	Roche Modular Elecsys	0.2–3.4	0.4–3.6	0.4–4.2	12–22	10–18	10–18	3.5–6.3	3.3–5.7	3.3–5.8
Cotzias et al. [28]	All trim Cross-sectional	ADVIA Centaur System	0–5.5	0.5–3.5	0.5–4.0	10–16	9–15.5	8–14.5	3–7	3–5.5	2.5–5.5
Dhatt et al. [32]	All trim/Arabs Cross-sectional	Abbott Architect	0.06–8.3	0.17–5.9	0.2–6.9	8.9–24.6	8.4–19.3	8.0–18.0	ND	ND	ND
Dhatt et al. [32]	All trim/Asians Cross-sectional	Abbott Architect	0.12–7.4	0.3–5.5	0.3–4.9	11.3–21.9	9.7–18.5	8.9–16.6	ND	ND	ND
Gilbert et al. [31]	1st trim	Abbott Architect	0.02–2.2	ND	ND	10–17.8	ND	ND	3.3–5.7	ND	ND
Gong and Hoffman [34]	All trim Cross-sectional	Roche Modular Elecsys	ND	ND	ND	11–19	9.7–17.5	8.1–15.3	ND	ND	ND
Lambert-Messerlian et al.* [24]	1st and 2nd trim Longitudinal	Immulite 2000	0.1–2.7	0.4–2.8	ND	0.9–1.4	0.8–1.3	ND	ND	ND	ND
Larsson et al. [37]	All trim Longitudinal	Abbott Architect	0.1–3.4	0.4–3.4	0.4–4.0	ND	ND	ND	ND	ND	ND
La'ulu et al. [30]	2nd trim Cross-sectional	Abbott Architect i2000SR	ND	0.1–3.3	ND	ND	9.1–15.4	ND	ND	3.8–6.0	ND
Marwaha et al. [29]	All trim Cross-sectional	Roche Modular Elecsys	0.6–5.0	0.4–5.8	0.7–5.7	12–19.5	9.5–19.6	11.3–17.7	1.9–5.9	3.2–5.7	3.3–5.2
Pearce et al. [36]	1st trim Cross-sectional		0.04–3.6	ND	ND		ND	ND	ND	ND	ND
Price et al. [33]	1st and 2nd trim/Asians Cross-sectional	Bayer Diagnostics ACS:180	0.6–1.3	1.0–1.8	ND	11.8–13.4	10.9–12.1	ND	ND	ND	ND
Price et al. [33]	1st and 2nd trim/Caucasian Cross-sectional	Bayer Diagnostics ACS:180	0.7–1.1	1.2–1.5	ND	12.0–12.8	11.2–11.8	ND	ND	ND	ND
Soldin et al. [35]	All trim Longitudinal	Tandem Mass Spectrometry	0.2–2.99	0.5–3.0	0.4–2.8	3.7–23.4	7.4–18.9	8.3–15.6	ND	ND	ND

TSH was measured in mU/L, free T4 in pmol/L, and free T3 in pmol/L. ND: not done. * the 5th and 95th percentile; free T4 in μg/L.

Table 2: What to do in clinical practice concerning thyroid function tests in pregnancy, when diagnosing hypo- or hyperthyroidism, respectively?

(i) Hypothyroidism:
(a) Serum TSH, evaluation respecting the gestation-induced suppression
(b) Measurement of antithyroperoxidase antibodies
(c) Sometimes measurement of TSH receptor antibodies and/or thyroglobulin antibodies
(d) Free T4 estimate
(1) "Direct measurement" with difficulty of interpretation
(2) Measurement of total T4 and T3 uptake test—more reliable
(3) Measurement of total T4 and correcting by 50% increase for pregnancy/TBG effect
(ii) Hyperthyroidism:
(a) Serum TSH, evaluation respecting the gestation-induced suppression
(b) Measurement of TSH receptor antibodies
(c) Free T4 estimate/free T3 estimate
(1) "Direct measurement" with difficulty of interpretation
(2) Measurement of total T4/T3 and T3 uptake test—more reliable
(3) Measurement of total T4/T3 and correcting the nonpregnant reference range by 50% increase for pregnancy/TBG effect

Adapted from: [10].

autoantibodies in this normal population. However, only antithyroperoxidase antibodies were significantly associated with hypothyroidism (as well as with hyperthyroidism), whereas antithyroglobulin antibodies were not [42].

7. Conclusions

In conclusion, in clinical practice doctors should use all available thyroid function tests relevant for the diagnosis of the AITD in question (see Table 2) to avoid the risk of false interpretations and the resulting potential irreversible damage to the foetal brain. Both total and free thyroid hormones are liable to false results during pregnancy, and the biochemical panel of measurements performed when there is a suspicion of hypo- and hyperthyroidism should therefore be supplemented with antithyroperoxidase antibodies, in case of suspected hypothyroidism [42], and TSH receptor antibodies, in case of suspected hypo- or hyperthyroidism, respectively [43]. TSH is insufficient as sole and first-line diagnostic variable due to the thyroid-pituitary instability during pregnancy and to suppression during the high peak of HCG at the end of the first trimester. Only women without thyroperoxidase antibodies should be included when producing trimester specific reference ranges. The finding of low intraindividual variation of the thyroid hormones in serum would speak in favour of using the woman's own evolution of the thyroid hormones when diagnosing hypo- or hyperfunction. Since this is for obvious reasons not always possible, the use of trimester-specific references may at least serve to reduce the variability and improve diagnostics.

Access to a broad spectrum of thyroid function tests must be considered a prerequisite for taking proper care of pregnant women with AITD. Due to the high TBG concentration, the best laboratory assessment of thyroid function also in AITD is a free thyroid hormone estimate—in hypothyroidism a free T4 estimate combined with TSH and in hyperthyroidism free T4 and T3 estimates combined with TSH. These free thyroid hormone measurements do not always correct completely for the binding protein abnormalities. Thus, if in doubt, samples should be measured in another laboratory with different platforms for free thyroid hormone measurements or combined with total hormone measurement and a T3 uptake test. Measurement of antithyroperoxidase, antithyroglobulin, and/or TSH receptor antibodies will add to the differential diagnosis between AITD and nonautoimmune thyroid disease. Thus, presence of TPO antibodies very often predicts the risk of hypothyroidism, and, in pregnant women with low TSH, hyperthyroidism will be predicted by TSH receptor antibodies in 60–70%.

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Case Report

A Patient with Postpartum Hypopituitarism (Sheehan's Syndrome) Developed Postpartum Autoimmune Thyroiditis (Transient Thyrotoxicosis and Hypothyroidism): A Case Report and Review of the Literature

Nobuyuki Takasu and Yoshirou Nakayama

Department of Endocrinology and Metabolism, Aizawa Hospital, 2-5-1 Honjo, Mtasumoto 390-8521, Japan

Correspondence should be addressed to Nobuyuki Takasu, nobuyukitakasu@gmail.com

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A 36-year-old woman with postpartum hypopituitarism (Sheehan's syndrome: SS) developed postpartum autoimmune thyroiditis (PPAT). She delivered a baby by Caesarean section (620 mL blood loss). At 1 month post partum, she developed thyrotoxicosis due to painless thyroiditis (autoimmune destructive thyroiditis). She was positive for antithyroid antibodies. Postpartum and hypoadrenalism-induced exacerbation of autoimmune thyroiditis caused the thyrotoxicosis due to autoimmune destructive thyroiditis. ACTH was undetectable. She had ACTH deficiency and secondary hypoadrenalism. Hydrocortisone was started. At 6 months post partum, she was referred to us with hypothyroidism. Thyroxine was administered. She had thyrotoxicosis at 1-2 months post partum and then hypothyroidism. She was diagnosed with PPAT. She had hypopituitarism, ACTH deficiency (secondary hypoadrenalism), low prolactin with agalactia, and low LH with failure to resume regular menses. She had empty sella on MRI. She was diagnosed with SS. Three cases with SS have been reported to develop PPAT. Postpartum immunological rebounds and hypoadrenalism-induced immunological alterations (or a combination of the two) might have been responsible for the PPAT.

1. Introduction

Sheehan's syndrome (SS), first described by Sheehan in 1937 [1], is postpartum hypopituitarism caused by intrapartum or postpartum hemorrhage. SS may cause partial or complete hypopituitarism. It may also cause secondary hypoadrenalism. Pregnancy and delivery have a profound effect on autoimmune thyroid diseases during gestation and the postpartum period [2]. Postpartum transient thyrotoxicosis and hypothyroidism have been reported [3, 4]. They are postpartum exacerbation or development of autoimmune thyroiditis and have been called postpartum autoimmune thyroiditis (PPAT), postpartum thyroiditis, or postpartum painless thyroiditis [3–8]. PPAT, postpartum thyroiditis, or postpartum painless thyroiditis is a member of autoimmune thyroiditis (Hashimoto's thyroiditis) [9]. The exacerbation and development of autoimmune thyroiditis have also been reported after adrenalectomy in patients with Cushing's syndrome [10, 11]. The decrease in cortisol after adrenalectomy exacerbates autoimmune thyroiditis. Exacerbation of autoimmune thyroiditis has been also reported after cessation of steroid therapy in a patient with autoimmune thyroiditis and rheumatoid arthritis [12]. Three cases with SS have been reported to develop PPAT [5, 13, 14]. A case with transient thyrotoxicosis due to painless thyroiditis (autoimmune destructive thyroiditis) following pituitary apoplexy was also reported [15]. Pituitary apoplexy and SS may cause secondary hypoadrenalism or a serum cortisol decrease. This decrease in cortisol may exacerbate autoimmune thyroid diseases. Steroid hormones decrease after delivery. Postpartum steroid hormone decrease may exacerbate autoimmune thyroid diseases.

We encountered a patient with postpartum hypopituitarism (Sheehan's syndrome: SS), who developed postpartum autoimmune thyroiditis (PPAT) (transient thyrotoxicosis and hypothyroidism). Postpartum immunological rebounds and hypoadrenalism-induced immunological alterations (or a combination of the two) might have been responsible for the development of PPAT in this patient.

2. Materials and Methods

2.1. Hormone Assays. Serum TSH, free T3, free T4, total T3, total T4, thyroglobulin, antithyroid peroxidase antibody (TPOAb), antithyroglobulin antibody (TGAb), progesterone, estradiol, serum prolactin, and plasma ACTH were determined by electrochemiluminescence immunoassays (ECLIA) (Roche Diagnostics, Tokyo, Japan). The intraassay coefficient of variation (CV) was 2.1%, 3.5%, 5.2%, 4.3%, 3.2%, 5.1%, 5.1%, 6.5%, 4.5%, 3.3%, 3.1%, and 3.6%, respectively, and interassay CV was 3.5%, 8.4%, 9.4%, 9.4%, 8.2%, 7.8%, 9.4%, 10.6%, 9.2%, 6.4%, 6.5%, and 7.2%, respectively. Serum TSH receptor antibody (TRAb) (TRAb (human)) was determined by a radioreceptor assay (RRA) (Yamasa Co., Tokyo, Japan). The intraassay CV was 7.6%, and interassay CV was 12.4%. Serum cortisol, GH, IGF-1, and urinary cortisol were measured by radioimmunoassay (RIA) (TFB, Inc., Tokyo, Japan). The intraassay CV was 5.8%, 3.3%, 3.0%, and 6.8%, respectively, and interassay CV was 8.9%, 6.5%, 6.2%, and 10.2%, respectively. LH and FSH were measured by chemiluminescence immunoassay (CLIA) (Abbott Lab., Tokyo, Japan). The intraassay CV was 3.5% and 3.3%, respectively, and interassay CV was 6.5% and 7.2%, respectively. Plasma ADH was measured by RIA (Mitsubishi Chemical Medicine Corp., Tokyo, Japan). The intraassay CV was 6.1%, and interassay CV was 9.5%. Hormone assays were performed at the SRL Institute (Tokyo, Japan). Normal reference ranges for hormone concentrations are described in the tables, legends for figures, or elsewhere as cited.

2.2. Endocrine and Other Studies. A thyrotropin-releasing hormone (TRH) test, using 500 μg TRH, was performed to estimate TSH and prolactin secretion. The test was done in the morning after an overnight fast. Samples for TSH and prolactin were drawn at 0, 30, 60, 90, and 120 minutes after intravenous TRH administration. Peak TSH and prolactin levels occur at 30 minutes in normal subjects.

A corticotrophin-releasing hormone (CRH) test, using 100 μg CRH (human CRH: Corticorelin), was performed to estimate ACTH secretion. The test was done in the morning after an overnight fast. The patient was on bed rest for at least 40 minutes before the first blood sample was drawn. Plasma ACTH and serum cortisol levels were measured at 0, 30, 60, 90, and 120 minutes after the intravenous CRH injection. Peak ACTH levels occur at 30–60 minutes in healthy subjects, while a lack of ACTH secretion is seen in patients with pituitary ACTH insufficiency.

A gonadotropin-releasing hormone (GnRH) test, using 100 μg GnRH (LH-RH), was performed to estimate LH and FSH secretion. The test was done in the morning after an overnight fast. Samples for LH and FSH were drawn at 0, 30, 60, 90, and 120 minutes after the intravenous GnRH injection. Gonadotropin deficiency was diagnosed by subnormal LH and FSH responses to GnRH. TRH, CRH, and GnRH tests were performed separately.

An insulin tolerance test (ITT) was used to test growth hormone (GH) secretion. The test was performed in the morning after an overnight fast. Five-unit insulin was given intravenously, and glucose and GH concentrations were measured at −30, 0, 30, 60, 90, and 120 minutes. GH deficiency was defined by a peak GH response of less than 3 μg/L with low concentrations of IGF-I [16].

Written informed consent was obtained from the patient prior to publication of this paper.

3. Case Report

A 36-year-old Japanese woman was referred to us at 6 months post partum with easy fatigability and agalactia (Figure 1 and Table 1, 6 months). She had delivered a full-term baby by Caesarean section (Figure 1, Delivery). Her blood-loss was estimated to be 620 mL. During the delivery, she did not have hypotension and remained normotensive. She was discharged without any apparent complications. However, she began to complain of easy fatigability, lassitude, agalactia, and loss of appetite after the delivery.

She visited a doctor at 1 month post partum (Figure 1 and Table 1, 1 month). A physical examination at that time revealed a supine blood pressure of 90/48 mmHg. Her pulse rate was 122/min and temperature was 37.2°C. An examination showed moist skin and finger tremors with clear lungs and a soft abdomen. Exophthalmoses were not observed. She had thyrotoxicosis clinically. A thyroid function study demonstrated that she had thyrotoxicosis; serum free T3 and free T4 levels were elevated, and serum TSH levels were undetectable (Figure 1 and Table 1, 1-2 months). She was negative for TRAb. However, she was positive for TPOAb and TGAb. At 1 month ante partum, her TPOAb was 3.4 kIU/L and her TGAb was 52.0 kIU/L (Table 1). At 2 months post partum, her TPOAb had increased to 42.2 kIU/L and her TGAb had increased to 138.4 kIU/L. Her serum thyroglobulin was 72 μg/L (normal < 32 μg/L), radioactive iodine uptake was 0.5%/24 hr (normal 10–40%), and thyroid scanning with radioiodine showed no detectable uptake. She had thyrotoxicosis due to autoimmune destructive thyroiditis. Her plasma ACTH was less than 0.4 pmol/L, and her serum cortsol was less than 5.5 nmol/L. She therefore had ACTH deficiency and secondary hypoadrenalism; 20 mg hydrocortisone (HC) was started (Figure 1, 1 month post partum). The thyrotoxicosis, due to autoimmune destructive thyroiditis, subsided spontaneously. She became euthyroid. At 4 months post partum, she had hypothyroidism with a serum TSH of 6.6 mIU/L (Figure 1 and Table 1, 4 months).

At 6 months post partum, she was referred to us with easy fatigability and agalactia (Figure 1 and Table 1, 6 months). On admission, she was well oriented and fully conscious. Her height was 163 cm, and her weight was 52.7 kg. She was afebrile with a temperature of 36.5°C. Her blood pressure

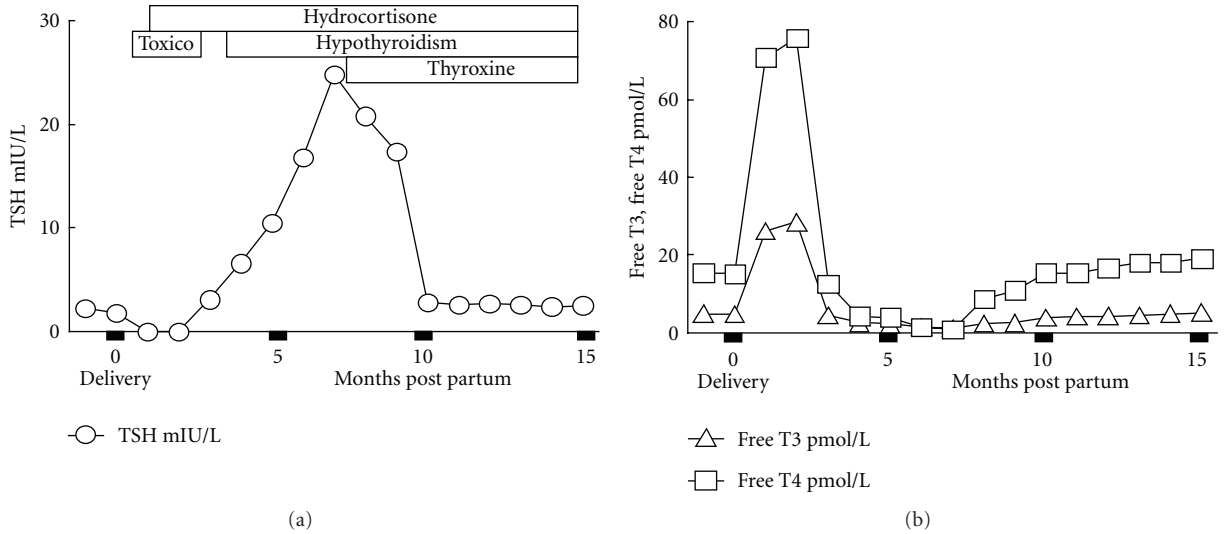

Figure 1: The clinical course of a patient with postpartum hypopituitarism (Sheehan's syndrome: SS), who developed postpartum autoimmune thyroiditis (PPAT) (transient thyrotoxicosis and hypothyroidism). A 36-year-old woman delivered a full-term baby by Caesarean section (Delivery). At 1 month post partum, she visited a doctor with thyrotoxicosis (Toxico). She was negative for TRAb. However, she was positive for TPOAb and TGAb. TPOAb- and TGAb-titers increased after delivery. Her serum thyroglobulin was 72 μg/L (normal < 32 μg/L). Radioactive iodine uptake was 0.5%/24 hr (normal 10–40%). She had thyrotoxicosis (Toxico) due to painless thyroiditis (autoimmune destructive thyroiditis). Her ACTH was less than 0.4 pmol/L, and her cortisol was less than 5.5 nmol/L. She had ACTH deficiency and secondary hypoadrenalism; 20 mg hydrocortisone (HC) was started. The thyrotoxicosis subsided spontaneously. At 4 months post partum, she developed hypothyroidism (hypothyroidism) with TSH 6.6 mIU/L. At 6 months post partum, she was referred to us with easy fatigability and agalactia. She had hypothyroidism with TSH 16.8 mIU/L. She had thyrotoxicosis (Toxico) at 1-2 months post partum and then hypothyroidism (hypothyroidism) (PPAT). At 7 months, thyroxine (T4) was started. She had hypopituitarism and empty sella on MRI (SS). She is now taking 75 μg T4 and 20 mg HC daily. Normal reference ranges: TSH 0.4–4.20 mIU/L, free T3 (free triiodothyronine) 3.5–6.6 nmol/L, and free T4 (free thyroxine) 11.6–21.9 pmol/L.

Table 1: Results of thyroid and adrenal function tests and TPOAb and TGAb at 1 month before delivery (−1 m) (1 month ante partum) and 1–10 months after delivery (1–10 m) (1–10 months post partum).

Months (m)*	−1 m	1 m	2 m	4 m	6 m	7 m	8 m	9 m	10 m
Free T3 pmol/L	4.9	26.2	28.5	2.5	1.5	1.2	2.3	2.5	3.9
Free T4 pmol/L	15.4	70.8	75.9	4.3	1.4	0.9	8.7	10.9	15.4
TSH mIU/L	2.2	<0.005	<0.005	6.6	16.8	24.8	20.8	17.4	2.8
ACTH pmol/L	11.5	<0.4	<0.4**		<0.4**				
Cortisol nmol/L	690	<5.5	<5.5**		<5.5**				
TPOAb kIU/L	3.4		42.2		34.1				26.8
TGAb kIU/L	52.0		138.4		126.7				102.3
TRAb IU/L	0.3		0.4		0.5				0.4

Months (m)*: −1 m: 1 month before delivery (1 month ante partum) and 1–10 m: 1–10 months after delivery (1–10 months post partum). **Oral hydrocortisone (HC) had been discontinued for 1 week before the study. Free T3: free triiodothyronine, free T4: free thyroxine, TPOAb: antithyroid peroxidase antibody, TGAb: antithyroglobulin antibody, and TRAb: TSH receptor antibody. Normal reference ranges: free T3 3.5–6.6 nmol/L, free T4 11.6–21.9 pmol/L, TSH 0.4–4.20 mIU/L, ACTH 1.70–12.27 pmol/L, cortisol 110–505 nmol/L, TPOAb < 0.3 kIU/L, TGAb < 0.3 kIU/L, and TRAb (TRAb (human)) < 1.0 IU/L.
A patient with postpartum hypopituitarism (SS) developed postpartum autoimmune thyroiditis (PPAT). At 1 month post partum, she had thyrotoxicosis. She was negative for TRAb. She was positive for TPOAb and TGAb. TPOAb- and TGAb-titers increased after delivery. Serum thyroglobulin was 72 μg/L (normal < 32 μg/L). She had painless thyroiditis (autoimmune destructive thyroiditis). She had thyrotoxicosis due to destructive thyroiditis. ACTH was less than 0.4 pmol/L and cortisol was less than 5.5 nmol/L. She had ACTH deficiency and secondary hypoadrenalism; HC was started. At 4 months, she had hypothyroidism with TSH 6.6 mIU/L. At 6 months, TSH was 16.8 mIU/L. At 7 months, thyroxine (T4) was started. She had thyrotoxicosis at 1-2 months post partum and then hypothyroidism (PPAT). She had hypopituitarism (SS). She is now taking T4 and HC.

was 100/64 mmHg. Her pulse rate was 72/min. Her skin was not moist. She had a goiter and delayed deep tendon reflexes. She had sparse axillary and pubic hairs. She had atrophy of the breasts and agalactia. She had also failed to resume regular menses after delivery. Systemic examination did not indicate any other abnormalities. She had hypothyroidism with a serum TSH of 16.8 mIU/L (Figure 1 and Table 1, 6 months). Figure 1 shows her clinical course. Since she had thyrotoxicosis at 1-2 months post partum and then developed hypothyroidism, she was diagnosed to have postpartum

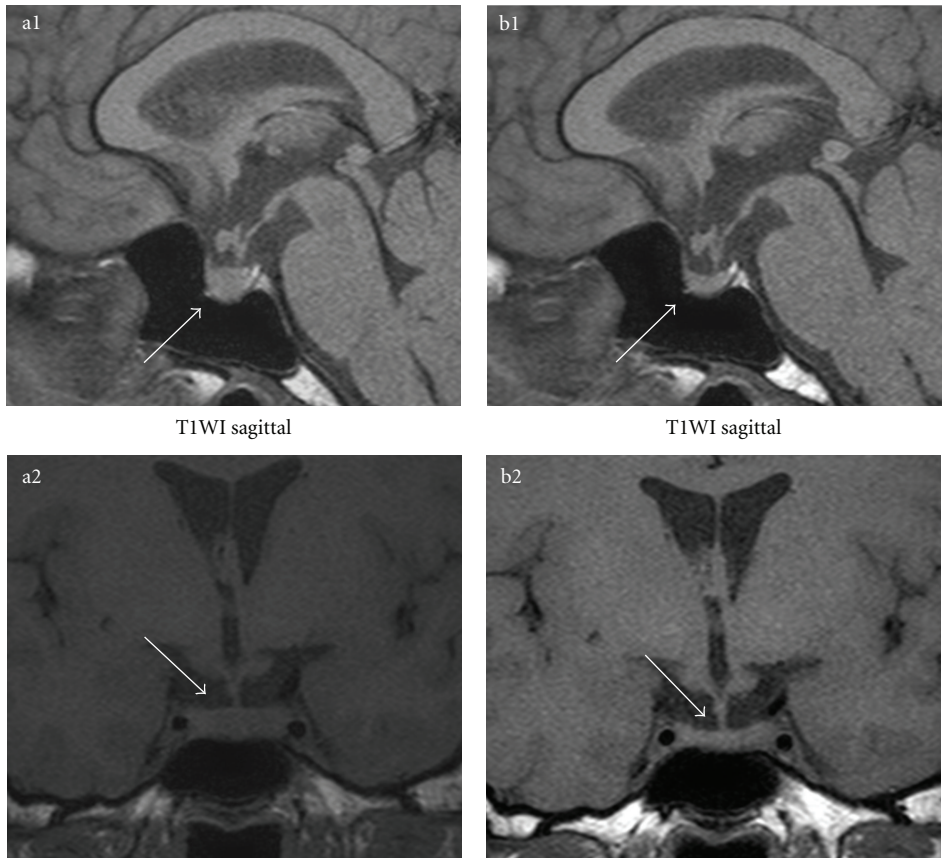

Figure 2: Sequential magnetic resonance imaging (MRI) (T1 weighted image: T1WI) demonstrated changes of the pituitary gland. At 2 months post partum, MRI revealed a normal pituitary gland ((a1) T1WI sagittal, (a2) T1WI coronal). At 6 months post partum, MRI revealed atrophy of the pituitary gland and empty sella ((b1) T1WI sagittal, (b2) T1WI coronal). At 6 months post partum, she had empty sella on MRI. The arrows indicate the pituitary gland.

autoimmune thyroiditis (PPAT). At 7 months post partum, thyroxine (T4) was started. She is now taking 75 μg T4 and 20 mg HC daily.

The patient had adrenal insufficiency. The laboratory evaluation at 6 months post partum had revealed that she had low plasma ACTH, low serum cortisol (Table 1, 6 months, Table 2), and low urinary cortisol (Table 3(a)). Table 3(b) demonstrates that she had no ACTH response to CRH. She therefore had ACTH deficiency and secondary hypoadrenalism.

She had agalactia. She had low serum prolactin (Tables 2 and 3(b)) and a low prolactin response to TRH (Table 3(b)). She had failed to resume regular menses after delivery. She had low LH (Tables 2 and 3(b)) and delayed LH and FSH responses to GnRH (Table 3(b)).

At 6 months, she had hypothyroidism with a serum TSH of 16.8 mIU/L (Figure 1 and Table 1, 6 months, Table 2). The elevated TSH levels indicated that she had primary hypothyroidism. She was positive for TPOAb and TGAb. She therefore had primary hypothyroidism due to autoimmune thyroiditis. Her TSH levels were high, her TSH response to TRH was delayed, and the magnitude of the TSH response to TRH was low (Table 3(b)), indicating that the pituitary TSH response to TRH might be impaired. It is possible that she had pituitary TSH secretion failure due to SS, in addition to primary hypothyroidism due to autoimmune thyroiditis [17].

The GH response to ITT was blunted (Table 3(c)) and the IGF-1 level was in the lower normal range (Table 2). The patient therefore had partial GH deficiency. She did not have diabetes insipidus (Table 3(d)).

The patient was determined to have hypopituitarism, ACTH deficiency (secondary hypoadrenalism), low prolactin with agalactia, and low LH with failure to resume regular menses. Sequential magnetic resonance imaging (MRI) demonstrated changes in the pituitary gland. At 2 months post partum, MRI had revealed a normal pituitary gland (Figure 2(a)), while, at 6 months, MRI revealed atrophy of the pituitary gland and empty sella (Figure 2(b)).

In summary, a patient with postpartum hypopituitarism (Sheehan's syndrome: SS) developed postpartum autoimmune thyroiditis (PPAT). She had hypopituitarism (ACTH

deficiency, low prolactin, and low LH) and empty sella on MRI (SS). She developed transient thyrotoxicosis at 1-2 months post partum and then subsequent hypothyroidism (PPAT).

4. Discussion

A patient with postpartum hypopituitarism (Sheehan's syndrome: SS) developed postpartum autoimmune thyroiditis (PPAT). After delivery, she had agalactia and failure to resume regular menses and was found to have hypopituitarism and empty sella on MRI (SS). She had transient thyrotoxicosis at 1-2 months post partum and then developed hypothyroidism (PPAT). Postpartum immunological rebounds and hypoadrenalism-induced immunological alterations (or a combination of the two) may have been responsible for the development of PPAT.

SS may cause secondary hypoadrenalism. SS is characterized by a wide spectrum of clinical features. In the past, hypothyroidism in the postpartum period was considered in the context of hypopituitarism due to SS. However, studies over the past several decades have altered this concept. Exacerbation or development of autoimmune thyroid diseases has been reported to occur among women after uneventful delivery and has been called postpartum autoimmune thyroiditis (PPAT), postpartum thyroiditis, or postpartum painless thyroiditis (postpartum autoimmune destructive thyroiditis) [3–8]. PPAT, postpartum thyroiditis, or postpartum painless thyroiditis is a member of a group of autoimmune thyroiditis (Hashimoto's thyroiditis) [9].

PPAT may involve thyrotoxicosis or hypothyroidism. The first phase is typically thyrotoxicosis due to autoimmune destructive thyroiditis (painless thyroiditis). Then, the thyroid function returns to normal. Some patients may subsequently develop hypothyroidism. Three cases with postpartum hypopituitarism (SS) have been reported to develop postpartum autoimmune thyroiditis (PPAT) [5, 13, 14].

The criteria for the diagnosis of SS are as follows: (1) typical obstetric history of intrapartum or postpartum bleeding, (2) hypotension or shock, (3) agalactia, (4) failure to resume regular menses after delivery, (5) hypopituitarism, and (6) empty sella on CT or MRI [18]. Our patient had (3), (4), (5), and (6). Kaplun et al. [19] reported that sequential MRI demonstrates evidence of ischemic infarct in the pituitary gland with enlargement, followed by gradual shrinkage to pituitary atrophy. In our case, MRI revealed atrophy of the pituitary gland and empty sella at 6 months post partum.

SS is described as postpartum hypopituitarism due to pituitary necrosis caused by hypotension or shock secondary to massive bleeding during or just after delivery. The exact pathogenesis and natural history are not understood [18]. The role of autoimmunity, including pituitary autoimmunity, in the development of SS has been also suggested, since pituitary autoantibody positivity is significantly higher in SS patients than controls [20, 21]. In SS, pituitary CT or MRI reveals an empty sella, similar to our patient.

Table 2: Fasting blood hormone levels at 9:00 (6 months post partum)*.

	Fasting hormone levels at 9:00(normal references)
ACTH pmol/L	<0.4 (1.7–12.3)
Cortisol nmol/L	<5.5 (110.4–504.9)
TSH mIU/L	16.8 (0.4–4.20)
fT3 pmol/L [tT3 nmol/L]	1.54 (3.54–6.62) [0.97 (1.23–2.46)]
fT4 pmol/L [tT4 nmol/L]	1.42 (11.58–21.88) [12.9 (78.5–159.6)]
Prolactin pmol/L	104 (266–1328)
GH μg/L	1.0 (0.28–1.64)
IGF-1 μg/L	112 (73–311)
LH IU/L	0.7 (1.13–14.22)
FSH IU/L	2.9 (1.47–8.49)
Progesterone nmol/L	98.5 (4.07–98.8)
Estradiol pmol/L	607.6 (165.2–1101.3)

*Oral hydrocortisone (HC) had been discontinued for 1 week before the study. fT3: free triiodothyronine, tT3: total triiodothyronine, fT4: free thyroxine, and tT4: total thyroxine.

Komatsu et al. [22] reported that serum antipituitary antibodies were positive in 70% of patients with empty sella and suggested that antipituitary antibodies might be related to the development of pituitary atrophy and the consequent empty sella. Our patient had positive tests for TPOAb and TGAb, indicating that she had had autoimmune thyroiditis. Both obstetric hemorrhage and autoimmune processes may affect postpartum hypopituitarism. Immune process might also be involved in the development of SS [20, 21].

Postpartum autoimmune thyroiditis (PPAT) is believed to result from the modifications of the immune system during pregnancy and delivery. PPAT may involve thyrotoxicosis and/or hypothyroidism. The first phase is typically thyrotoxicosis due to autoimmune destructive thyroiditis. Patients with PPAT have positive TPOAb and/or TGAb. They may have recurrences after each pregnancy and may eventually develop hypothyroidism. Any woman who develops PPAT should be carefully followed up, as she has an increased risk of developing hypothyroidism.

Three cases with postpartum hypopituitarism (SS) have been reported to develop postpartum autoimmune thyroiditis (PPAT) [5, 13, 14] (Table 4). We herein reported another case of SS, who developed PPAT. All four cases had positive TPOAb and/or TGAb. They initially presented with transient thyrotoxicosis due to autoimmune destructive thyroiditis. Three of the four patients were Japanese. The postpartum changes of autoimmune system may be responsible for the development of PPAT. SS also causes secondary hypoadrenalism, which causes a decrease in cortisol. The cortisol decrease can then induce immunological alterations. Postpartum immunological rebounds and hypoadrenalism-induced immunological alterations (or a combination of the two) might therefore be the underlying pathogenesis

Table 3: Endocrine studies at 6 months post partum.

(a) Urinary cortisol (studied one week after discontinuation of hydrocortisone)

	(Normal references)
Urinary cortisol nmol/day	<18 (30–230)

(b) CRH, TRH, and GnRH tests (CRH, TRH, and GnRH tests were done separately)

	0 min	30 min	60 min	90 min	120 min	Response
CRH test (studied one week after discontinuation of hydrocortisone)						
ACTH pmol/L	<0.4	<0.4	<0.4	<0.4	<0.4	No
Cortisol nmol/L	<5.5	<5.5	<5.5	<5.5	<5.5	No
TRH test						
TSH mIU/L	17.4	38.1	52.9	57.1	57.7	Delayed
Prolactin pmol/L	43.5	178.3	160.9	165.2	139.1	Low
GnRH test						
LH IU/L	0.5	6.6	11.1	13.1	13.7	Delayed
FSH IU/L	2.2	2.6	3.3	3.9	4.3	Delayed

Normal references for basal ACTH, cortisol, TSH, prolactin, LH, and FSH appear in Table 2.

(c) Insulin tolerance test (ITT) for GH releases

	−30 min	0 min	30 min	60 min	90 min	120 min
GH μg/L	1.0	1.1	0.8	4.1	3.8	2.5
BG nmol/L	4.6	4.3	2.2	3.1	3.7	4.3

BG: blood glucose. Normal references for basal GH appear in Table 2.

(d) ADH (antidiuretic hormone), plasma osmolality, and urine osmolality at 9:00

	(Normal references)
Plasma ADH pmol/L	0.92 (0.28–3.23)
Plasma osmolality mmol/kg	287 (285–293)
Urine osmolality mmol/kg	767 (300–900)

Table 4: Postpartum hypopituitarism (Sheehan's syndrome: SS) and postpartum autoimmune thyroiditis (PPAT): a review of the literature.

Case	Age, yr	Nationality	ATA*	Thyroid state**	Publication year	Reference
Case 1	38	Japanese	Positive	Thyrotoxicosis	1992	[12]
"Simultaneous occurrence of postpartum hypopituitarism (Sheehan's syndrome) and transient resolving thyrotoxicosis due to postpartum painless thyroiditis"***						
Case 2	29	Japanese	Positive	Thyrotoxicosis	1997	[13]
"Painless thyroiditis developed in a patient with Sheehan's syndrome"***						
Case 3	30	French	Positive	Thyrotoxicosis	2002	[4]
"Postpartum autoimmune thyroiditis in a patient presenting with Sheehan's syndrome"***						
Case 4	36	Japanese	Positive	Thyrotoxicosis		
"A Patient with postpartum hypopituitarism (Sheehan's Syndrome) developed postpartum autoimmune thyroiditis (PPAT) (transient thyrotoxicosis and hypothyroidism)"***						

ATA*: antithyroid antibodies (antithyroid peroxidase antibody (TPOAb) and/or antithyroglobulin antibody (TGAb)). Thyroid state**: the patient initially presented with thyrotoxicosis. ***: title of the report. Cases 1, 2, and 3 have been reported previously [4, 12, 13], respectively. Case 4 is presented in this paper.

for the development of PPAT. A case of SS with Graves' hyperthyroidism has been also reported [23].

Glucocorticoids are the main endogenous anti-inflammatory agents in vivo, interfering with every step of immune and inflammatory responses [24], and are commonly used in the treatment of autoimmune diseases. The development of autoimmune thyroid diseases has been reported after unilateral adrenalectomy for Cushing's syndrome [10], after bilateral adrenalectomy in a patient with Carney's complex [11] and after removal of ACTH-producing pituitary adenoma in patients with Cushing's disease [11]. The importance of endogenous glucocorticoids in the control of immune conditions is also exemplified by the increased mortality associated with adrenalectomy in rats

with experimental allergic encephalomyelitis [25]. Transient thyrotoxicosis was also reported to occur after cessation of steroid therapy in a patient with autoimmune thyroiditis and rheumatoid arthritis [12]. Another patient with hypopituitarism, following pituitary apoplexy, developed transient thyrotoxicosis due to painless thyroiditis (autoimmune destructive thyroiditis) [15]. Pituitary apoplexy and SS cause ACTH deficiency and secondary adrenocortical insufficiency (a cortisol decrease). This cortisol decrease may exacerbate autoimmune thyroid diseases. Hypoadrenalism induces immunological alterations, which may be associated with the development of PPAT.

In summary, we experienced a patient with SS who developed PPAT. Postpartum immunological rebounds and hypoadrenalism-induced immunological alterations (or a combination of the two) may have been responsible for the development of PPAT.

Conflict of Interests

None of the authors have accepted any funding or support from any organization that may gain or lose financially from the results of our study. None of the authors have been employed by any organization that may gain or lose financially from the result of our study.

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Clinical Study

Occurrence of Type 1 Diabetes in Graves' Disease Patients Who Are Positive for Antiglutamic Acid Decarboxylase Antibodies: An 8-Year Followup Study

Matsuo Taniyama,[1] Akira Kasuga,[2] Chieko Nagayama,[1] and Koichi Ito[3]

[1] *Division of Endocrinology & Metabolism, Department of Internal Medicine, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama, Kanagawa 227-8501, Japan*
[2] *Department of Internal Medicine, Tokyo Denryoku Hospital, 9-2 Shinanomachi, Shinjuku-ku, Tokyo 160-0016, Japan*
[3] *Ito Hospital, 4-3-6 Jingumae, Shibuya-ku, Tokyo 150-8308, Japan*

Correspondence should be addressed to Matsuo Taniyama, taniyama@med.showa-u.ac.jp

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Glutamic acid decarboxylase antibodies (GADAs) are one of the markers of islet cell autoimmunity and are sometimes present before the onset of type 1 diabetes (T1D). GADA can be present in Graves' patients without diabetes; however, the outcome of GADA-positive Graves' patients is not fully understood, and the predictive value of GADA for the development of T1D in Graves' patients remains to be clarified. We investigated the prevalence of GADA in 158 patients with Graves' disease and detected GADA in 10 patients. They were followed up to discover whether or not T1D developed. In the course of eight years, 2 patients with high titers of GADA developed T1D, both had long-standing antithyroid drug-resistant Graves' disease. Thus, Graves' disease with high GADA titer seems to be at high risk for T1D.

1. Introduction

Autoimmune type 1 diabetes (type 1A diabetes) is an organ-specific autoimmune endocrine disease, which is caused by immune destruction of pancreatic β cells [1]. Antibodies to islet-related antigens including glutamic acid decarboxylase antibodies (GADAs) and insulinoma-associated antigen 2 (IA-2) antibodies are markers for autoimmunity to islet cells [2, 3]. When these antibodies are positive, the patient's diabetes is usually considered to be type 1 even if they are not insulin dependent [4–6]. Antibodies to islet-related antigens are present before the onset of type 1 diabetes (T1D) [7], and their predictive value for the development of T1D has been repeatedly investigated in close relatives of T1D patients and the general population [7–12].

Graves' disease, which is also an organ-specific autoimmune endocrine disease, is frequently associated with T1D [13]. In these patients, titers of GADA tend to be high [14], which may indicate powerful ability of producing autoimmune process to islet antigens. On the other hand, GADA is sometimes positive in Graves' patients without diabetes [15–17]. In these patients, GADA may exist independently from β-cell destruction. However, the fate of Graves' patients who are positive for GADA is obscure, and the predictive value of GADA for the development of T1D in Graves' patients remains to be clarified. We examined GADA in patients with Graves' disease and followed up patients who were positive for GADA for 8 years.

2. Patients and Methods

GADA was measured by a highly sensitive ligand-binding assay in 158 patients with Graves' disease (50 untreated, 108 treated) who had not been diagnosed to have diabetes. The patients were randomly collected by one physician at Ito

Thyroid Clinic. Most patients other than new patients were under the treatment with antithyroid drugs. In the patients who were positive for GADA by ligand-binding assay (positive when detected), GADA was again measured by radioimmunoassay (Cosmic Corporation, standard value was <1.5 U/ml), and antibodies to Islet Cell Antibodies (ICAs) 512/IA-2 were measured by a ligand-binding assay (positive when detected). Details of the ligand-binding assay for GADA and antibodies to ICA512/IA-2 have been described elsewhere [18, 19]. Glucose intolerance was assessed by either oral glucose tolerance test or HbA1c (reference range: 4.3–5.8%) within a half year after the detection of GADA. Diabetes was diagnosed by criteria of American Diabetes Association. In the cases in which only HbA1c was measured for the detection of glucose intolerance, less than 5.8% was considered to be normal glucose tolerance. Patients positive for GADA by ligand-binding assay were followed up whether or not type 1 diabetes developed. In seven patients whose GADA titers by ligand-binding assay were relatively high and GADA by RIA were positive, HbA1c and occasional plasma glucose were measured at least every two years for eight years. GADA was occasionally measured by radioimmunoassay. In other three patients who were negative for GADA by RIA, physicians inquired whether or not diabetes developed at every visiting to the outpatient clinic. One patient was dropped out three years after the initial workup. The patients who were negative for GADA at the start of the study were not followed up.

3. Results

Ten patients out of 158 (6.3%) were positive for GADA by the ligand-binding assay. Eight of these patients were treated with antithyroid drugs (ATDs) and 2 were untreated (treatment naive). The overall prevalence of positivity for GADA among treated and untreated patients was 7.4% and 4.0%, respectively (Table 1). GADA was again investigated by standard radioimmunoassay (RIA) in 9 of the 10 patients, and 6 were positive (Table 2). In 4 patients, titers by RIA were over 20 U/ml. ICA512/IA-2 antibodies were weakly positive in 2 patients. An oral glucose tolerance test was performed in 5 of the 10 GADA-positive patients. Of these, one patient showed a diabetic pattern and another had impaired glucose tolerance (this patient dropped out from study 3 years after the initial workup). BMI of these patients was 19.9 and 21.0, respectively. The other 3 patients had normal GTT. HbA1c levels of the other 5 patients were within the normal range. During the 8-year followup period, T1D developed with marked hyperglycemia and ketosis in two patients whose Graves' disease was long standing and uncontrollable by antithyroid drug. One of them showed diabetic pattern in GTT at the initial work up, but HbA1c was within a normal range (Section 3.1 and Table 2).

3.1. Case Reports. Patient 1 was a 44-year-old man (at the diagnosis of T1D), in whom Graves' disease developed at age 21. He took an antithyroid drug (ATD), but 10–20 mg of methimazole was needed to maintain euthyroid. At age 42, GADA was detected. His oral glucose tolerance test the next year showed a diabetic pattern (FPG 7.8 mmol/L (141 mg/dL), 2 hours 14.3 mmol/L (257 mg/dL)). HbA1c was 5.7%. His insulin response to oral glucose was very low (insulinogenic index, 0.08). GADA by RIA was as high as 6090 U/ml. Calorie restriction was recommended, but symptoms of severe hyperglycemia including thirst and polyuria developed the following year. Plasma glucose was 29.6 mmol/L (534 mg/dL), and urine ketone bodies were 2+. Blood gas analysis did not demonstrate acidosis (pH 7.385). Insulin therapy was started, and the requirement of insulin was reduced to 2 units/day but increased to 34 to 40 units thereafter. Postprandial C-peptide reactivity (CPR) was 0.23 nmol/L at 3 years after the onset of diabetic ketosis. Finally, the patient received semitotal thyroidectomy after the exacerbation of thyrotoxicosis.

Table 1: Prevalence of GAD antibodies in Graves' patients who had not been diagnosed with diabetes at the study start.

	Patient number	GADA(+)	(%)
Graves' disease	158	10	6.3
untreated	50	2	4.0
treated	108	8	7.4

Difference of prevalence is not statistically significant.

Patient 2 is a 34-year-old woman (at the diagnosis of T1D). Her grandmother had type 2 diabetes. Graves' disease developed at age 14. After ATD therapy, she had remission at age 19, but Graves' disease relapsed at age 21. At age 26, Graves' disease was exacerbated 9 months after delivery. GADA was detected next year. Postprandial glucose was 5.3 mmol/L (95 mg/dL), and HbA1c was 5.0%. GADA by RIA was 855 U/ml. Three years later, Graves' disease was exacerbated again after her second delivery. She needed 60 mg methimazole to maintain euthyroid and she took radioisotope therapy, but an antithyroid drug was continued as she was still thyrotoxic. One year after RI therapy, her postprandial plasma glucose was 123 mg/dL and HbA1c was 5.6%. GADA by RIA was increased to 1440 U/ml. Next year T1D developed with the manifestations of hyperglycemia such as thirst, polydipsia, polyuria, and weight loss. On the laboratory examinations, plasma glucose was 34.6 mmol/L (623 mg/dL), HbA1c was 14.1%, urine ketone bodies were positive, and arterial blood pH was 7.41. After initial therapy for hyperglycemia, she took 24 units of insulin daily, and her CPR before lunch was 0.18 nmol/L.

4. Discussion

Type 1 diabetes (T1D) and Graves' disease, both endocrine organ-specific autoimmune diseases, frequently coexist and in combination are classified as autoimmune polyglandular syndrome type III [13]. There are common genetic backgrounds for both diseases [20] such as the CTLA-4 gene [21–23] and PTPN-22 gene [24–27]. In Japanese adults, the two diseases often develop simultaneously or Graves' disease proceeds to T1D [28]. Thus, Graves' disease is a risk factor for T1D, as seen in the present study in which two out

Table 2: Baseline characteristics of the patients with positive GAD antibodies including their antibody titers.

Case		Age at entry	Sex	duration (yr)	GADA (LBA) 0	GADA (RIA) u/ml <1.5	IA-2Ab 0	TRAb (%) <10.0	TGHA (X) <400	MCHA (X) <400	HbA1c (%) 4.3–5.8	GTT
1	*	42	M	21	>1.300	6090	0.024	63.5		400000	5.7	DM
2		21	F	0	>1.128	>256	neg			400		normal
3	*	27	F	13	0.928	855	neg	21.5		400000	5.0	
4		50	F	11	0.790	21.4	neg	7.2	400	100000		IGT
5		26	F	0	0.192	2.6	neg	6.7	1600	25600		normal
6		23	F	5	0.076		neg	24.2		100000		
7		25	F	4	0.073	3.3	neg	29.5		400000	4.4	normal
8		42	F	2	0.062	0.5	neg	21.1			4.9	
9		25	M	6	0.047	<0.4	0.083	7.0		6400	3.9	
10		42	F	12	0.027	<0.4	neg	12.5			4.8	

Figures at the bottom of the items are reference values.
*Cases 1 and 3 developed T1D.
neg: negative.
Age: age at examination of GAD antibodies.
TRAb: TSH receptor antibodies, TGHA: thyroglobulin hemagglutination, MCHA: microsome hemoagglutination.

of 158 patients with Graves' disease developed T1D during the course of 8 years.

In this study, the prevalence of GADA in Graves' patients without previously diagnosed diabetes was high, similar to those in previous reports [15–17]. Furthermore, the prevalence was high in the treated patients but the difference of the prevalence between treated patients and treatment-naïve patients was not statistically significant. Among the patients positive for GADA, T1D developed in two patients with long-standing Graves' disease who were not easily controlled by antithyroid drug (ATD) therapy. One needed a 10 to 20 mg methimazole for control of the disease and finally had a thyroidectomy after exacerbation. The other frequently relapsed and finally had radioiodine therapy. Their titers of GADA both by ligand-binding assay and radioimmunoassay were very high. It is conceivable that autoimmune reaction to islet antigens is strong in the Graves' patients with high titer of GADA, and that those patients are susceptible to T1D.

Antibodies to islet antigens are present before the onset of type 1 diabetes [7]. On the other hand, many individuals positive for antibodies to islet antigens do not develop T1D. In Finland, where the incidence of T1D is very high, it was reported that T1D developed only in 26% of GADA-positive young subjects from general population over 25 years [29]. In the same study, only 0.26% of GADA-negative subjects developed T1D. Previous studies have revealed that positivity for more than 2 kinds of islet-associated antibodies, especially the combination of GADA and IA-2 antibodies, has predictive value [2]. Of the patients in the present study who developed T1D, one had IA-2 antibodies and the other did not. On the contrary, one patient who had both antibodies in low titers did not develop T1D during the followup period. The number of patients was small and we could not obtain conclusive results, but the presence of both GADA and IA-2 antibodies seems to indicate a high risk also in Graves' patients. Screening of GADA followed by examination of IA-2 antibodies may allow detecting those patients at greater risk for development of T1D, and careful followup may provide earlier detection of the onset of T1D in these patients.

5. Conclusions

We show preliminarily that Graves' patients with long duration and high titers of GADA are at high risk for developing T1D. To clarify what factors are involved in the susceptibility to T1D in Graves' disease, greater numbers of patients need to be followed up intensively over a long period of time.

Abbreviations

GADA: Glutamic acid decarboxylase antibodies
T1D: Type 1 diabetes
IA-2: Insulinoma-associated antigen 2
CPR: C-peptide reactivity
ICA: Islet cell antibodies
ATD: Antithyroid drug
RIA: Radioimmunoassay.

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Review Article

Clinical and Pathological Implications of Concurrent Autoimmune Thyroid Disorders and Papillary Thyroid Cancer

L. L. Cunha,[1] R. C. Ferreira,[1] M. A. Marcello,[1] J. Vassallo,[2] and L. S. Ward[1]

[1] *Laboratory of Cancer Molecular Genetics, Faculty of Medical Sciences, University of Campinas (FCM-Unicamp), 126 Tessalia Vieira de Camargo St., Cidade Universitária, Barão Geraldo, Campinas, 13083-970 São Paulo, SP, Brazil*
[2] *Laboratory of Molecular Pathology, Ciped, Faculty of Medical Sciences, University of Campinas (Unicamp), 126 Tessalia Vieira de Camargo St., Cidade Universitária, Barão Geraldo, Campinas, 13083-970 , São Paulo, SP, Brazil*

Correspondence should be addressed to L. S. Ward, ward@unicamp.br

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Cooccurrences of chronic lymphocytic thyroiditis (CLT) and thyroid cancer (DTC) have been repeatedly reported. Both CLT and DTC, mainly papillary thyroid carcinoma (PTC), share some epidemiological and molecular features. In fact, thyroid lymphocytic inflammatory reaction has been observed in association with PTC at variable frequency, although the precise relationship between the two diseases is still debated. It also remains a matter of debate whether the association with a CLT or even an autoimmune disorder could influence the prognosis of PTC. A better understanding about clinical implications of autoimmunity in concurrent thyroid cancer could raise new insights of thyroid cancer immunotherapy. In addition, elucidating the molecular mechanisms involved in autoimmune disease and concurrent cancer allowed us to identify new therapeutic strategies against thyroid cancer. The objective of this article was to review recent literature on the association of these disorders and its potential significance.

1. Introduction

Thyroid cancer is the most common endocrine malignancy and was considered responsible for 1600 deaths amongst 300,000 patients diagnosed with the disease in the USA in 2009 [1]. The incidence of differentiated thyroid cancer (DTC) in the United States and worldwide continues to increase, having more than doubled over the past three decades [2, 3]. A similar trend has been observed in other countries across Europe, Asia, Oceania, and South America [4]. In fact, only a few countries have reported a decline in thyroid cancer incidence: Sweden (18% reduction for both men and women), Norway (5.8% reduction for women), and Spain (25.9% reduction for women) [4]. The disease incidence is increasing in other European countries, such as Switzerland (5.3%) and France (155.6%) [4]. A common aspect in all epidemiological reports is that the highest rate of increase is for small and localized thyroid cancers, which probably contributes to the stable and relatively low mortality observed. However, there are several lines of evidence indicating that the increased incidence of DTC is also related to other factors, including the increasing amount of large tumors that would certainly be detected without the need for more sophisticated or sensitive imaging methods and a parallel increase of exposure to a series of environmental factors associated with cancer, such as exposure to radiation [5, 6]; living in volcanic areas [7]; iodine intake [8, 9]; female gender [10], which might be related to estrogen, a subject that is being studied for our group; obesity [11]; and genetic factors that might be related to susceptibility to DTC [12–17].

A parallel increase has been observed in the incidence of autoimmune thyroid diseases, such as thyroiditis. Chronic lymphocytic thyroiditis (CLT) and DTC, mainly PTC, share some epidemiological features, such as the relationship with ionizing radiation exposure [6, 18] and dietary iodine [19, 20]; both also share some molecular features [21–28] and are more likely to occur in women than in men [29–31].

Although most tumors originate from follicular cells, they present remarkably different features. In addition, likewise breast, prostate, and other human neoplasias, microscopic thyroid tumors are frequently found at autopsies and in surgical specimens [32]. However, the impact of the immune system, the presence of autoimmune thyroid diseases, and their relationship with cancer development is a matter of controversy [4]. Also, literature repeatedly reports association between CLT and thyroid cancer. Geographically widespread association and incidence of CLT with thyroid cancers are shown on Table 1.

2. Concurrent Chronic Lymphocytic Thyroiditis (CLT), Thyroid Autoimmune Diseases, and Differentiated Thyroid Carcinomas (DTC)

Hashimoto's disease and Graves' disease are the two most common forms of autoimmune thyroiditis (AT), the archetypal organ-specific autoimmune disease in humans. Both are characterized by lymphocytic infiltrate and autoreactivity against thyroid autoantigens [43–45].

Chronic lymphocytic thyroiditis (CLT) is an autoimmune disease characterized by widespread lymphocyte infiltration, fibrosis, and parenchymal atrophy of thyroid tissue. Hashimoto's thyroiditis (HT) is characterized by infiltration of the thyroid gland by inflammatory cells, often followed by hypothyroidism due to destruction and eventual fibrous replacement of the parenchymal tissue. In HT, the body also produces autoantibodies to thyroid-specific antigens (Figure 1), considering that thyroglobulin (Tg) and thyroperoxidase (TPO) are the two primary antigens in AT [46–48]. HT is characterized by a gradual loss of thyroid function, goiter, and T-cell infiltration in histology, affecting women more often than men, with a sex ratio of 7 : 1, and occurring in genetically susceptible populations, but lacking a strong association with HLA. The overriding feature of HT is the progressive depletion of thyroid epithelial cells, which are gradually replaced by mononuclear cell infiltration and fibrosis [47, 49, 50]. In thyroiditis, especially HT, parenchyma of thyroid gland is progressively lost and replaced by cells of the inflammatory infiltrate that produce chemokines, cytokines, and growth factors, most of which are under NF-β transcriptional control. The persistent stimulation of residual thyrocytes with such molecules could induce the activation of NF-β in follicular cells, thereby creating a functional network between thyroid epithelial cells and inflammatory cells [49].

In a number of human malignancies, the presence of lymphocytic infiltration in or around a tumor is commonly viewed as representing a host immune response [52]. Although the presence of tumor-associated lymphocytic infiltration is widely regarded as representing a host immune response, the impact of this inflammatory response on tumor behavior may be variable [28, 53–55]. In addition to the common occurrence of CLT and papillary thyroid carcinomas (PTC), it has been suggested that the relatively high prevalence of apparently indolent PTC in an autopsy series may represent host immune control [56].

The relationship between CLT and PTC was first proposed by Dailey et al. in 1955 [21]. Since this initial description, the association between the diseases has been repeatedly reported and highly debated in the literature, remaining controversial.

A thyroid lymphocytic inflammatory reaction has been observed in association with PTC at variable frequency, ranging from 0.5% to 58% [21–28, 34, 37, 39, 40, 57, 58]. This wide distribution of coexisting CLT and PTC reported in a number of studies may be due, at least in part, to differences in the level of histological examination and criteria of autoimmunity characterization, patient selection or indications for thyroidectomy, environmental factors (history of radiation exposure) [59], genetic or population background [57], and geographic factors (e.g., the amount of iodine intake) [59–61].

Thyroid autoimmunity is a broad spectrum disease also manifested as the presence of antithyroid antibody (ATA). A series of reports indicate a close association between ATA and malignancy [50, 62], while others are not able to confirm this association [63–66]. Boi et al. assessed ultrasound- (US-) guided fine-needle aspiration cytology obtained from 590 unselected consecutive patients with single thyroid nodules and positive or negative serum antithyroid antibody. Cytological results were divided into three classes of increased risk of malignancy: low risk or benign, indeterminate risk, and suspect or malignant. They suggested that the presence of antithyroid antibodies confers an increased risk of suspicious or malignant cytology in unselected thyroid nodules [62]; even this result is not confirmed in other reports [67, 68].

There are reports in the literature on the relationship between other thyroid autoimmune diseases, such as Graves's disease and thyroid cancer. Some studies have suggested a high incidence of malignant thyroid nodules in patients with Graves' disease and hyperthyroidism, and that thyroid cancer behaves more aggressively when associated with Graves' disease, although still controversial [69–72]. As TSH stimulates growth of metastatic differentiated thyroid cancer expressing the TSH receptor (TSHR), it is possible to hypothesize that high levels of anti-TSHR antibodies of Graves' patients might stimulate thyroid cancer growth and early metastatic spread, thus negatively affecting patient outcome, as reviewed by Belfiore et al. [73]. However, other studies do not support the suggestions that thyroid cancer in patients with Graves' disease is more aggressive than in either patients with toxic nodular goiter or euthyroid subjects, suggesting that concurrent Graves disease is not a good prognosticator [74, 75].

Even in reduced frequency, CLT is also presented in association with follicular thyroid carcinoma (FTC). Loh et al. [37] studied subjects with lymphocytic thyroiditis (LT), including Hashimoto's thyroiditis and cell lymphocytic infiltrates around thyroid neoplasms. Lymphocytic thyroiditis (LT) was recorded in 125 of 564 patients (22%) with PTC histology, when comparing with three of 67 patients (4.5%) with follicular or Hürthle cell histology. They found that patients with LT almost uniformly had PTC, consistent with the observation by other investigators [33, 34, 76, 77]. In fact, Souza et al. [38] reported that TPO

Table 1: Last twenty years of published results. Autoimmune manifestation was significantly correlated with the presence of follow-up features, disease presentation features, and individual features.

Reference	Autoimmune manifestation	Type of thyroid cancer	Number of patients	Follow-up features	Disease presentation features	Individual features	Geographic widespread	Concurrent CLT in thyroid cancer (%)
[33]	Lymphocytic infiltration	PTC	95	Relapse-free	Circulating autoantibodies, coexisting thyroid disease	None	Japan	37.89
[34]	Chronic lymphocytic thyroiditis	PTC	1533	Relapse-free, overall survival	None	None	Japan	18.09
[35]	Chronic lymphocytic thyroiditis	PTC	69	None	Multifocality	None	Japan	21.74
[36]	Lymphocytic infiltration	PTC+FTC+ MTC+ATC	153	None	Low pT stage	None	Germany	17.65
[37]	Both Hashimoto's thyroiditis and lymphocytic infiltration	PTC+FTC	631	Low cancer recurrence rate, low cancer mortality rate	Low extrathyroidal invasion, nodal metastases, distant metastases, pTNM stage	Female	USA	20.28
[28]	Hashimoto's thyroiditis and lymphocytic infiltration separately	PTC	136	Overall survival	Multifocality, Tumor Infiltrating lymphocytes	Young, female	USA	30.15
[38]	Circulating autoantibodies and history of autoimmune thyroid disease	PTC+FTC	173	Relapse-free, overall survival	None	None	Brazil	8.67% (+TPO*Ab*) + 6.94% (+Tg*Ab*) + 13.87% (with Graves or Hashimoto history)
[39]	Hashimoto's thyroiditis	PTC	101	None	None	Old	Korea	36.6
[40]	Chronic lymphocytic thyroiditis	PTC	1441	Relapse-free	Small tumors, low pTNM stage	Female	Korea	14.85
[41]	Lymphocytic infiltration and/or circulating autoantibodies	PTC	343	None	None	None	Italy	37.31
[42]	Chronic lymphocytic thyroiditis	PTMC	323	None	TgAb positive, Microsomal Ab positive, multifocality, and bilaterality	Female	Korea	32.51

PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; PTMC: papillary thyroid microcarcinoma; MTC: medullary thyroid carcinoma; ATC: anaplastic thyroid carcinoma; Ab: antibody.

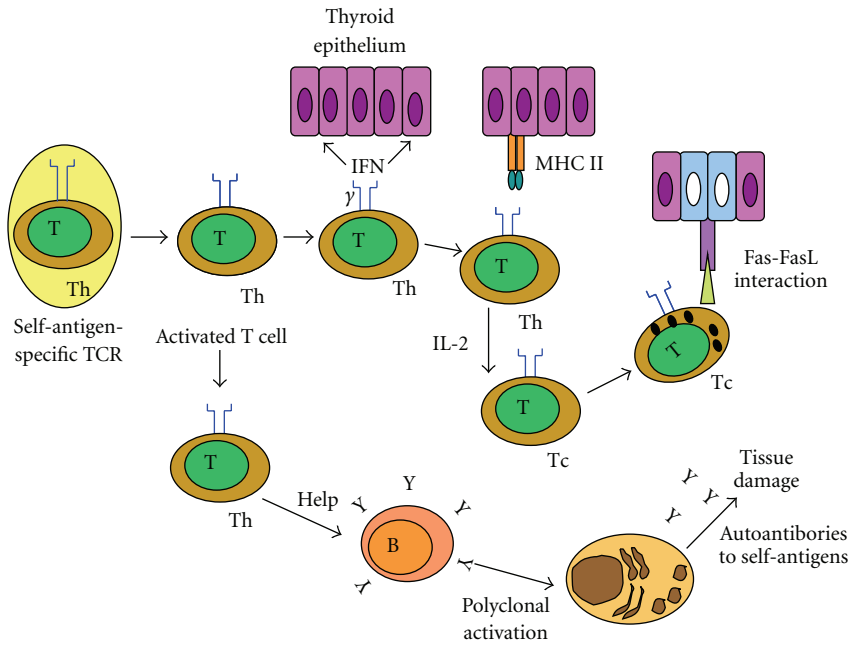

Figure 1: Activation of a self-specific T-cell initiates a cascade of events that amplifies the immune response and involves both CD4 and CD8 T cells, inducing an antibody-mediated response. B: B cells; IFN-γ: interferon-γ; IL-2: interleukin-2; MHCII: major histocompatibility class II; T: T cells; Tc: cytotoxic T-cell; Th: T helper cell (adapted from [51]).

antibody was present in 14 of 123 patients (11.3%) with PTC histology, when comparing with one of 50 patients (2%) with follicular or Hürthle cell histology. In addition, thyroglobulin antibody (TgAb) was present in 12 of 123 patients (9.76%) with PTC, while none of FTC patients presented TgAb at diagnosis, confirming the correlation between concurrent autoimmunity and PTC histology.

3. Molecular Link between Thyroid Cancer and Chronic Lymphocytic Thyroiditis

A functional relationship between chronic inflammation and cancer was first proposed by Virchow, in 1863, and has been sustained by clinical [41, 78] and epidemiological evidence [79–82]. Both a causal association and a noncausal association have been proposed, and the molecular mechanism that links inflammation and cancer is not completely clear so far. A link between thyroid cancer, in particular the PTC histotype, and AT has long been recognized, although the precise relationship between the two diseases remains subject of debate.

Hashimoto's thyroiditis is characterized by proliferating nodules as well as by cytological alterations and nuclear modifications similar to those of papillary carcinomas, suggesting that both neoplastic and autoimmune diseases could share the same molecular pathogenesis [83].

The mitogen-activated protein kinase (MAPK) signaling pathway is a foremost event in the carcinogenesis of the most common endocrine malignancy, the papillary thyroid carcinoma (PTC). Affected elements include RET/PTC rearrangements and point mutations of the *RAS* and *BRAF* genes. Mutations in these genes are found in over 70% of PTC, as previously reviewed [84]. Chromosomal RET rearrangements, called RET/PTC, result in constitutive ligand-independent activation of RET kinase, which was the first genetic anomaly detected in PTC and is found in 5–70% of tumor samples. Although less frequently, the activation of other tyrosine kinase receptors, including NTRK1, c-Met, or EGFR, has also been reported in PTC [85]. The *BRAF* mutation represents the most common genetic alteration found in PTC [86]. More than 90% of *BRAF* mutations lead to a change of valine to glutamic acid at position 600 (V600E) [85]. Finally, RAS is the least affected molecule in the pathway [85]. All of these multisteps of thyroid carcinogenesis are shown in Figure 2.

Several authors have found RET/PTC rearrangements in non-neoplastic thyroid lesions, such as CLT [88–90]. In addition, Muzza et al. found RET/PTC1 being more represented in PTCs associated with autoimmunity than in PTC without autoimmunity, suggesting that the association between RET/PTC1 and thyroiditis points to a critical role of this oncoprotein in the modulation of the autoimmune response [41]. Rhoden et al. showed that low-level RET/PTC recombination occurs in nonneoplastic follicular cells of HT and in a subset of papillary thyroid carcinomas, indicating that overlapping molecular mechanisms may govern early stages of tumor development and inflammation in the thyroid [91]. Kang et al. studied the RET/PTC-RAS-BRAF in oxyphil cells in the vicinity of large lymphoid HT infiltrates and in malignant PTC cells. The expression of RET, nuclear RAS, and ERK proteins is greatly enhanced in PTC cells and HT oxyphil cells. Thus, the RET/PTC-RAS-BRAF cascade may be involved in the development of PTC and oxyphil

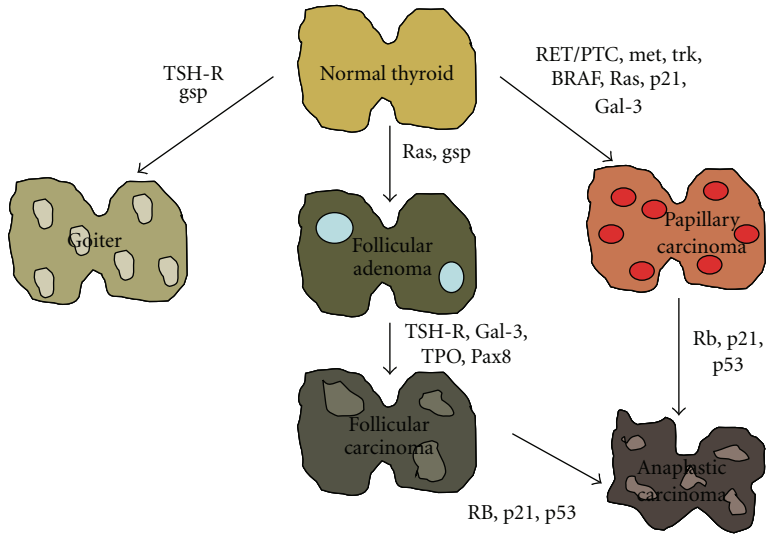

Figure 2: Multistep carcinogenesis model of thyroid cancer formation. Formation of benign thyroid tumors occurs as a result of alteration of various growth factors. Follicular neoplasms are formed from thyrocytes by mutations of RAS and other factors, as shown in the figure. Papillary cancers are formed by alterations in RET/PTC and other oncogenes. Undifferentiated tumors are formed from differentiated tumors by mutations of tumor suppressor genes (adapted from [87]).

cell metaplasia in HT. These results show the possibility of a molecular link between oxyphil cell metaplasia in HT and the progression of PTC [92].

However, different mechanisms could also explain the association between CLT and RET/PTC rearrangement (Figure 3). Chronic inflammation might facilitate the rearrangement or, conversely, RET/PTC rearrangement might promote chronic inflammation. The production of free radicals, cytokine secretion, cellular proliferation, and other phenomena correlated with inflammation might predispose to the rearrangement in follicular cells [93]. It is well known that leukocytes recruited under an inflammation context physiologically secrete reactive oxygen species and reactive nitrogen species. However, these highly reactive metabolites induce the production of peroxynitrite and other mutagenic agents, leading to "DNA damage", for example, mutations in proliferating cells [94]. Thus, in the case of persistent tissue damage, O_2 and N highly reactive metabolites secreted by inflammatory cells induce point-mutations, DNA rearrangements, and double-strand breaks [93, 95].

Guarino et al. proposed that cytokines and chemokines released by inflammatory tumor stroma could sustain the survival of thyroid cells in which RET/PTC rearrangements randomly occur, thereby allowing the selection of clones that acquire additional genetic lesions and thus become resistant to oncogene-induced apoptosis [93]. In fact, some reports indicate that RET/PTC might induce apoptosis [96, 97]. This hypothesis of interaction between RET/PTC rearrangement and thyroid inflammation is reinforced by some studies that suggest that thyroid cancer cells, like other epithelial cancer cells, can produce inflammatory factors that may facilitate cell survival, preventing apoptosis. Stassi et al. found that autocrine production of IL-4 and IL-10 promotes thyroid tumor cell progression and resistance to chemotherapy by the upregulation of antiapoptotic proteins, such as Bcl-2 and Bcl-xL [98]. Conticello et al. found that IL-4 protects tumor cells (primary prostate, breast, and bladder cancer) from CD95- and chemotherapy-induced apoptosis by the upregulation of antiapoptotic proteins, such as cFLIP/FLAME-1 and Bcl-x(L) [99]. Todaro et al. identified that primary epithelial cancer cells from colon, breast, and lung carcinomas produced interleukin-4 (IL-4), which amplified the expression levels of these antiapoptotic proteins and prevented cell death induced upon exposure to drug agents via downregulation of the antiapoptotic factors PED, cFLIP, Bcl-xL, and Bcl-2, providing evidence that exogenous IL-4 was able to upregulate the expression levels of these antiapoptotic proteins and potently stabilized the growth of normal epithelial cells making them apoptosis resistant [100].

On the contrary, RET/PTC rearrangement might induce chronic inflammation. Russell et al. found that RET/PTC3 alone increases nuclear NF-kappaB activity and secretion of MCP-1 and GM-CSF. Finally, transfer of RP3-expressing thyrocytes into mice in vivo attracted dense macrophage infiltrates, leading to rapid thyroid cell death [101]. In addition, the same group found that IL1-alpha, IL1-beta, IL6, TNF-alpha, and the Cox2 enzyme are produced by RET/PTC3-transgenic thyroid tissue, but absent from non-transgenic thyroids, providing support for the notion that oncogene-induced cytokine secretion is important for the development and progression of thyroid carcinomas in genetically permissive hosts [102]. Prostaglandin E2, microsomal prostaglandin E2, cyclooxygenase 2 (Cox2), IL24, and other genes coding for proteins involved in the immune response and in intracellular signal transduction pathways activated by cytokines and chemokines have been suggested to be induced by RET/PTC, indicating that the expression

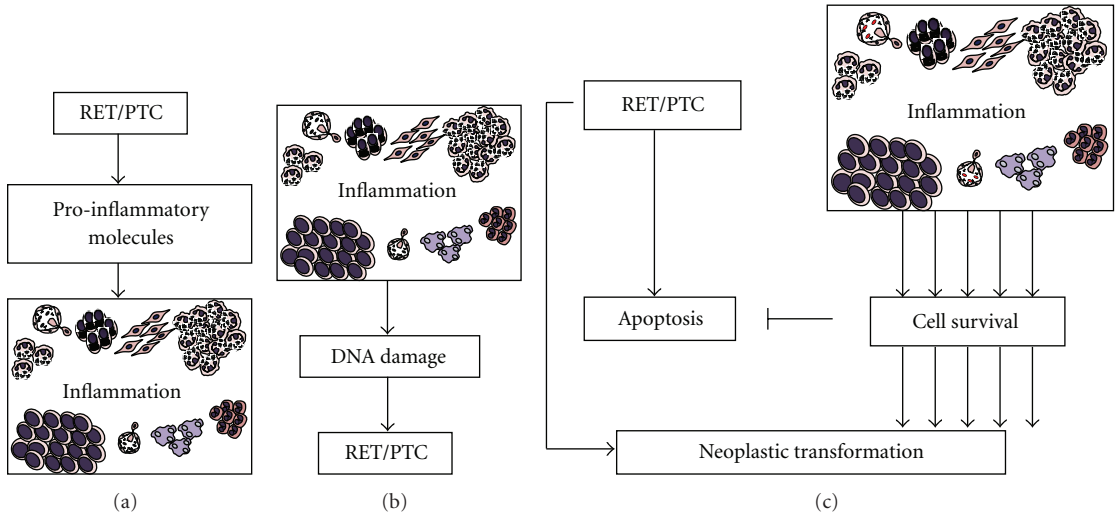

Figure 3: Putative links between RET/PTC rearrangement and concurrent thyroid inflammation. (a) RET/PTC could drive expression of several proinflammatory molecules that may elicit concurrent inflammation. Another possibility (b) is inflammation propitiating RET/PTC rearrangement. Inflammation produces reactive oxygen species and free radicals that may facilitate DNA damage and chromosomal abnormities, like RET/PTC rearrangement. (c) Molecules released by inflammation could sustain the survival of thyroid cells in which RET/PTC rearrangements randomly occur, thereby allowing the selection of clones that acquire additional genetic lesions and thus become resistant to oncogene-induced apoptosis.

of the oncogenic fusion protein RET/PTC is critical not only to thyroid cancer pathogenesis but also in the elicitation of inflammatory response [103–105].

BRAF^{V600E} mutation is another common genetic alteration in PTC. Muzza et al. found *BRAF*^{V600E} being more represented in PTC without concurrent autoimmunity [41]. Furthermore, Kim et al. found that, in Korean patients with PTC, *BRAF*^{V600E} mutation is associated with a lower frequency of background HT [39]. Studies on melanoma cells have given clues about mechanisms linking *BRAF* mutation and immune response. Molecules like interleukin (IL)-10, VEGF, IL-6, and IL-8 are thought to be induced by *BRAF*^{V600E} mutation [106, 107].

The association between solid cell nest (SCN) of the thyroid and both neoplastic and autoimmune thyroid diseases is the most recent and debated issue. It is currently accepted that solid cell nests and the so-called mixed follicles are branchial body remnants [108–116], whose biological significance remains disputable [108, 109, 117–119]. Previous studies have described the histological and immunohistochemical features of SCN [108, 109, 114, 117, 120–124]. Cameselle-Teijeiro et al. suggested that main cells of solid cell nests (SCNs) might be multipotential cells that could contribute to histogenesis of C cells and follicular cells, as well as to some thyroid tumors [119]. Recently, Preto et al. and Reis-Filho et al. reported that such main cells harbor the minimal properties of a stem cell phenotype (capacity for both self-renewal, conferred by telomerase activity, and differentiation to one or more types of specialized cells, given by the high expression of p63 and bcl-2) and may thus represent a pool of stem cells of the adult thyroid [123, 125]. Burstein et al. hypothesized that SCN p63-positive cells are pluripotent and may stay undifferentiated or undergo benign squamoid or glandular maturation, thyroid follicular epithelial differentiation, and oncogenic change leading to papillary carcinoma or may trigger an immune reaction, resulting in lymphoid infiltration and Hashimoto's thyroiditis. Hence, Hashimoto's thyroiditis and papillary carcinoma would be etiologically linked because both disorders might be initiated by the same population of pluripotent p63-positive embryonal stem cell remnants [122]. SCN could represent incompletely developed thyroid tissue predisposed to autoimmune thyroid diseases, such as Hashimoto's thyroiditis, since the epithelia from the third and the fourth pouches have the ability to attract lymphocytes [126, 127]. Cameselle-Teijeiro et al. described an unusual case of SCN hyperplasia coexisting with two papillary microcarcinomas, a follicular adenoma, hyperplastic nodules, and a few lymphoid aggregates [128]. A morphologic continuity between SCN and one papillary thyroid microcarcinoma was reported and the authors found the same *BRAF*^{V600E} mutation in both the SCN and the contiguous papillary thyroid microcarcinoma, suggesting a histogenetic link between the main cells of SCN and PTC, raising the possibility that SCN hyperplasia could be a precursor lesion of PTC [128]. These findings suggest that SCN may be a key point in the pathogenesis of both CLT and PTC.

Pathways of immune activation could exert a role in thyroid cancer and CLT link. Toll-like receptor (TLR) comprises a family of cell surface receptors involved in the recognition of pathogen-associated signature molecules that signal the activation of innate and adaptive immunity [129–133]. Although the TLR family consists of more than ten members [133], in humans, TLR3 had been reported to be restricted primarily to dendritic cells of the immune system

[134]. Harii et al. showed that TLR3 can be functionally overexpressed in cultured human thyrocytes by stimuli. Immunohistochemical showed that TLR3 protein is overexpressed in human thyrocytes surrounded by immune cells in 100% of patients diagnosed with Hashimoto's thyroiditis, but not in normal or Graves' thyrocytes, suggesting that TLR3 overexpression can induce an innate immune response in thyrocytes, which may be important in the pathogenesis of Hashimoto's thyroiditis and in immune cell infiltrates [129]. McCall et al. showed that PTC cells basally express TLR3 RNA and that TLR3 signal systems are functional in these cells. High basal TLR3 levels and TLR3 signals capable of increasing cytokines and chemokines in PTC cells in vitro are consistent with the existence of immune cell infiltrates in vivo, based on related studies suggesting that elevated TLR3/TLR3 signals in HT are associated with immune cell infiltrates [129, 135].

Studies on DNA damage and repair have recently yielded several intriguing connections between tumor biology and immune response. There is some evidence that ATM may exert a special role in the activation of the immune response. ATM is an essential component of cell cycle restriction point control. Its scope of interaction includes phosphorylation and activation of E2F1, p53, and Cdc25 family members, which inhibit cell cycle progression and activate DNA repair systems [136, 137]. Gomez et al. showed that ATM protein expression was significantly downregulated in immunoresistant human glioma cell clones [138], indicating a possible immunogenic role of ATM gene. DNA damage signaling can directly engage the immune system in a non-cell-type-specific manner. ATM activation by genotoxic agents or stalled DNA replication induces ligands of the NKG2D receptor. These are expressed by natural killer cells and activated CD8+ T cells of the innate immune system [139]. In addition, authors indicated that polymorphisms of DNA damage response genes, such as *ATM*, *XRCC1*, *TP53*, *XRCC3*, and *MTF1*, may be potential risk modifiers of ionizing radiation-induced or sporadic PTCs [140, 141]. Royer et al. reported that hOGG1, encoding human 8-oxoguanine DNA glycosylase (hOGG1), a key enzyme for repairing DNA damaged by reactive oxygen species and loss of heterozygosity, is strongly associated with PTC and HT but not with benign thyroid, suggesting that thyroid follicular epithelia accumulate aberrant genetic changes in long-standing HT, which may represent a precursor lesion of PTC [142]. In fact, data from our group showed a significant correlation between ATM expression and concurrent CLT in DTC (not published data).

4. Prognostic Implication of the Association between CLT and PTC

It remains a matter of debate whether the association with a CLT or even an autoimmune disorder could affect the prognosis of PTC. In fact, a worse prognosis was reported in a few series [72, 143], whereas most of the studies showed either a protective effect of thyroid autoimmunity [34, 37, 40, 144] or a similar behavior between cancer with and without associated thyroiditis [41, 74], as shown in Table 1.

Our group reported a worse outcome in DTC patients with no evidence of autoimmune activity when compared with patients who reported an autoimmune thyroid disease and/or presented positive circulating thyroid autoantibodies, suggesting that autoimmune activity against the gland may exert a protective effect on the outcome of differentiated thyroid carcinoma patients [38].

However, it is unclear whether coexistent CLT represents a host immune response to DTC [33, 60, 145] or just a chance occurrence [40, 146, 147]. The favorable clinical outcome in PTC patients with concurrent autoimmunity strongly suggests that a thyroid autoimmune response may enhance or even provide an antitumor attack. CLT is an autoimmune reaction to thyroid specific antigen, and this immune response may lead to destruction of thyroid tissue. Kim et al. [40] postulated that, as PTC cells originating from follicular cells expressed specific antigen of normal follicular cells, although in a less degree, coexistent CLT might be involved in destruction of tumor cells in much the same way as in advanced CLT. This immune reaction against tumor might be associated with a better prognosis for PTC patients with CLT. Studying the glands of autopsies performed in individuals from two different Brazilian regions (Rio Grande do Norte versus São Paulo), we found a high incidence of nonencapsulated nonsclerosing papillary thyroid microcarcinomas (PTM) in Rio Grande do Norte, an area of markedly high incidence of PTC, in sharp contrast with the prevalence of small sclerosing lesions in São Paulo, where clinical thyroid cancers are much more infrequent [148]. PTMs could elicit an inflammatory lymphocytic response, fibrosis, and the formation of a capsule impeding its further growth [148]. We could speculate that these lesions may never evolve to clinical cancers, in contrast with the nonencapsulated nonsclerosing lesions that could represent early stages of clinical PTC.

Conversely, antithyroid antibodies may be able to recognize these malignant cells and destroy them in the same way as they destroy normal follicular cells, contributing to the low rate of clinical progression of these lesions [32, 62, 149].

Muzza et al. [41] found no significant differences regarding either clinical and pathological features or outcomes between two matched groups of PTC patients with and without associated autoimmunity. The cases of PTC associated with lymphocytic thyroiditis (LT) are much more often multicentric than the nonassociated form of PTC [35]. Interestingly, those with multifocal tumors had a relatively high incidence of lymphocytic thyroiditis (62%) in the remaining thyroid parenchyma during the definitive histological examination [150, 151]. Kim et al. [42] found that in metastatic papillary thyroid microcarcinomas, multifocality and bilaterality were more frequent in PTC with LT than without LT. Kim et al. [39] found that a background of Hashimoto's thyroiditis is more frequent in young patients, who trend to be patients with good prognosis. However, they found that the $BRAF^{V600E}$ mutation in PTC is associated with a low frequency of background Hashimoto's thyroiditis and high frequency of lymph-node metastasis, suggesting a paradoxical role of concurrent autoimmunity of PTC outcome.

Some investigators have reported that the presence of CLT in PTC is associated with better prognosis, low recurrence rate, and less aggressive disease at presentation [33, 34, 36, 37, 40, 152, 153]. Despite some controversies, there is an emerging literature on the protective effect of CLT in patients with PTC. In a large retrospective study, Kashima et al. reported a 5% cancer-specific mortality and a 85% relapse-free 10-year survival rate in patients without CLT compared with 0–7% mortality and 95% relapse-free 10-year survival rate with CLT [34]; this finding was very similar to Loh's results [37]. It is, however, not clear whether treatment modality, including extent of surgery, was comparable between those with and without CLT. Kebebew et al. reported that the presence of CLT was associated with an improved prognosis by univariate analysis, but it was not an independent factor [28], and this result was due to the fact that most patients with CLT had other good prognostic factors, such as young age and female gender. Kim et al. found a greater female preponderance in the patients with CLT when comparing with those without CLT. Mean tumor size in the patients with CLT was smaller than that in patients without CLT. One hundred and fifty-one (12.3%) patients without CLT had recurrence, whereas 14 (7.1%) patients with CLT had recurrence during the follow-up period [40]. Singh et al. [30] reported that the prognostic variables at the time of a diagnosis of papillary cancer and the approach to management are not altered by the presence of coexistent Hashimoto's thyroiditis. In addition, the rate of surgical complications was not higher in patients with coexistent Hashimoto's disease, suggesting that the presence of coexistent Hashimoto's thyroiditis does not affect the diagnostic evaluation or management of papillary thyroid cancers. However, meta-analysis suggested a positive correlation between Hashimoto's disease and disease-free survival and overall survival [30].

5. Future Perspectives and Conclusion

Cooccurrences of CLT and thyroid gland cancer have been repeatedly reported [21–28]. A better understanding about clinical implications of concurrent autoimmunity to thyroid cancer could lead to new insights of thyroid cancer immunotherapy. In addition, elucidation of the molecular mechanisms involved in autoimmune disease and concurrent cancer could help identify new therapeutic strategies against thyroid cancer. In fact, within the last decade, a multitude of different studies and clinical trials have been performed using immune cell therapies for different cancers. Cancer immunotherapy using dendritic cells (DC) or adoptive cytotoxic T-lymphocytes (CTLs) is very promising because malignant cells can be affected by the immune system without damaging healthy tissue and without dangerous side effects. In any case, the identification of tumor cell-specific antigens is crucial for establishing clinically effective tumor immunotherapy and monitoring the induced immune response. Up to now, however, no single tumor-associated antigen has been proven useful for primary follicular thyroid carcinomas, although a couple of candidates might have this potential [154]. Only antibody-based therapies were performed on nonmedullary thyroid cancer, and there is a broad spectrum to be explored on cellular therapy against DTC, mainly for those cases with aggressive manifestation [155–157].

Medullary thyroid cancer (MTC) can give us an example for immunotherapy against thyroid cancer. The polypeptide hormone calcitonin has been proposed as tumor antigen for immunotherapy in MTC. Since then, several vaccination trials have been performed in murine models and also in men. In humans, several studies used full-length calcitonin for priming DCs [158, 159]. Thereafter, a new protocol with interferon-α generated DCs with direct tumor lysis activity was performed [160]. After a long-term follow-up of more than 48 months, two of five MTC patients showed stable disease with changes in tumor size and tumor marker of less than 25% [161]. More studies are warranted to apply all these concepts to poorly differentiated and anaplastic thyroid cancer.

Conflict of Interest

There is no financial interest in or arrangement with a company whose product was used in a study. In addition, there is no financial interest in or arrangement with a competing company, and there are no other direct or indirect financial connections or other situations that might raise the question of bias in the work reported or the conclusions, implications, or opinions stated—including pertinent commercial or other sources of funding for the individual author(s) or for the associated department(s) or organization(s), personal relationships, or direct academic competition.

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