

Thyrotropin Receptor Blocking Antibodies



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ABSTRACT

Autoantibodies (Ab) against the thyroid-stimulating hormone receptor (TSHR) are frequently found in autoimmune thyroid disease (AITD). Autoantibodies to the TSHR (anti-TSHR-Ab) may mimic or block the action of TSH or be functionally neutral. Measurement of anti-TSHR-Ab can be done either via competitive-binding immunoassays or with functional cell-based bioassays. Antibody-binding assays do not assess anti-TSHR-Ab functionality, but rather measure the concentration of total anti-TSHR binding activity. In contrast, functional cell-based bioassays indicate whether anti-TSHR-Ab have stimulatory or blocking activity. Historically bioassays for anti-TSHR-Ab were research tools and were used to study the pathophysiology of Graves' disease and Hashimoto's thyroiditis. In the past, bioassays for anti-TSHR-Abs were laborious and time-consuming and varied widely in performance from laboratory to laboratory. Recent advances in the development of cell-based assays, including the application of molecular engineering, have led to significant improvements that have enabled bioassays to be employed routinely in clinical laboratories. The prevalence and functional significance of TSHR blocking autoantibodies (TBAbs) in autoimmune hypothyroidism has been less well investigated compared to TSHR stimulating Ab. There is an increasing body of data, however, that demonstrate the clinical utility and relevance of TBAbs, and thus the importance of TBAbs bioassays, in the diagnosis and management of patients with AITD. In the present review, we summarize the different methods used to measure TBAbs, and discuss their prevalence and clinical relevance.

Abbreviations

AA	Amino acids	GD	Graves' disease
ATD	Antithyroid drugs	HT	Hashimoto's thyroiditis
Ab	Autoantibodies	IgG	Immunoglobulin G
anti-TSHR-Ab	Autoantibodies to the TSHR	L-T4	Levothyroxine
AITD	Autoimmune thyroid disease	LH-CG	Luteinizing hormone-choriogonadotropin
bTSH	Bovine TSH	MAB	Monoclonal antibody
CHO	Chinese hamster ovary	Mc4	Mutant chimeric 4
CREB	cAMP response element-binding protein	RAI	Radioactive iodine
cAMP	Cyclic adenosine 3',5'-monophosphate	RIA	Radio-immunoassay
FRTL-5	Fisher rat thyroid line-5	TBII	TSHR-binding inhibitory immunoglobulin
FDA	Food and Drug Administration	TBAbs	TSHR-blocking autoantibodies
		TSAb	TSHR-stimulating autoantibodies
		TSH	Thyroid-stimulating hormone

T4	Thyroxine
T3	Triiodothyronine
TSHR	TSH receptor
wt	Wild-type

Introduction

Autoimmune thyroid diseases (AITD) are characterized through increased familial clustering, reduced DNASE1 gene expression, CTLA-4 polymorphisms, and are strongly associated with the major histocompatibility (MHC) complex [1–4]. However, autoantibodies to various thyroid antigens are the most important biomarkers that are used to differentiate AITD from other thyroid conditions. Whereas antibodies to thyroid peroxidase (TPO) and thyroglobulin (Tg) neither play a major nor casual role in the pathophysiology of AITD, antibodies to the TSHR play a unique role in the development of autoimmune hyper- and hypothyroidism. Autoantibodies to the TSHR (anti-TSHR-Ab) are directly involved in the pathophysiology of Graves' disease (GD) and Hashimoto's thyroiditis (HT). GD is caused by TSHR-stimulating antibodies (TSAb), which act as agonists by stimulating thyroid growth and thyroid hormone synthesis in an unregulated manner [5–7]. In contrast, blocking anti-TSHR-Ab (TBAb) acts as TSHR antagonists, which block the action of the thyroid-stimulating hormone (TSH) and can cause the hypothyroidism of HT. Anti-TSHR-Ab can be detected either with immunoassays, which measure TSHR-binding inhibitory immunoglobulins (TBII) [8] or with cell-based bioassays, which measure either TSAb or TBAb [9]. The advantages and disadvantages of binding assays versus functional bioassays for anti-TSHR-Ab, with special emphasis on TBAb, are shown in ► **Table 1**.

Bioassays involve the measurement of signal transduction pathways mediated by binding of a ligand to the TSHR [10, 11]. Compared to TBII binding assays, cell-based bioassays are more sensitive in detecting low anti-TSHR-Ab concentrations and exclusively differentiate between the anti-TSHR-Ab functionality [12, 13]. Historically bioassays that measure TSAb were based on measurement

of cAMP levels in cells using radio-immunoassays (RIA) [14–19]. More recently, non-radioactive methods to measure cAMP have been used. In addition, cell lines that contain a cAMP-inducible reporter gene such as luciferase have been employed to measure TSAb [20–23]. Bioassays that measure TSHR-blocking activity are based on the same cell-based systems, but they detect the ability of patient antisera to block TSH or TSAb-stimulated cAMP levels or luciferase expression [23–25].

Most reviews of anti-TSHR-Ab have focused primarily on TSAb. In the present, paper we will focus on TBAb. Compared to TSAb [26–28], the measurement and clinical utility of functional TBAb have been less well investigated. Many reports have studied the occurrence of TBAb in various autoimmune diseases associated with hypothyroidism such as autoimmune-induced atrophic thyroiditis or primary myxedema [29–34]. The prevalence of TBAb in these various conditions is still controversial. Other investigations have focused on the role of TBAb in congenital and neonatal hypothyroidism associated with maternal AITD [31, 35–38]. The importance of TBAb in congenital hypothyroidism, however, has been questioned. Ever since it has been known that anti-TSHR-Ab exhibit different functional activities it has been suspected that certain patients might contain both TSAb and TBAb, and that this may explain certain clinical presentations. Recent evidence has proven that a patient can have both TSAb and TBAb by isolating separate monoclonal antibodies (MAB) with stimulatory and blocking activity from the lymphocytes of the same patient [39, 40]. In addition, there has been speculation that patients with GD may shift between stimulating and blocking antibodies as they transition from hyperthyroid to hypothyroid and vice versa. The mechanism of transition from TSAb to TBAb is most probably due to a different ratio between the two functional autoantibodies; while the exact antigenic sites of the TSHR against TBAb and TSAb are strongly overlapping and not completely defined. In the present paper, we will review the historical and currently used assays used for the measurement of TBAb activity and discuss their clinical applications.

► **Table 1** Advantages and disadvantages of binding (TBII) Assays versus functional bioassay for TSHR blocking autoantibodies.

	Advantages	Disadvantages
Binding Assays (TBII)	International standardization with reference material	Do not discriminate the functional antibody type
	Easy handling and performance	Only measure antibody binding to the TSHR
	M22 and KSA1, human and mouse thyroid stimulating mAbs respectively, have replaced bTSH in newer automated binding assays	TBII levels reflect total anti-TSHR-Ab
	Commercially automated assays are available	
Functional Bioassay	Measure the net sum of the functional activity	Absence of an international standard
	Discriminate the functional antibody type, specifically identifying TBAb*	More time consuming than TBII
	Higher analytical sensitivity than binding assays (TBII)	Requires experienced laboratory technician
	Newly developed bioassays: minimal handling of the cells, no IgG purification, no serum starvation, no serum concentration	Not widely available
	Predict fetal/neonatal risk for hypothyroidism in pregnant women with active or treated AITD	Not automated yet

* According to references [23, 25] and JP Banga, personal communication.

Pathophysiology of Hashimoto's Thyroiditis

Hashimoto's thyroiditis is the major cause of autoimmune hypothyroidism and GD is the primary cause of autoimmune hyperthyroidism. Whereas the hyperthyroidism of GD is exclusively due to TSAb, there are several mechanisms, by which hypothyroidism develops in HT. The primary pathological basis of decreased thyroid hormone production is related to immune-mediated apoptosis and cytolysis of thyrocytes. Normal thyroid epithelial cells express a variety of death receptors including Fas and activation of the Fas-ligand-Fas signaling system contributes to the follicular cell destruction characteristic of HT. In addition, cytokine stimulation from antigen-presenting cells and Th1 cells (IL-1) can induce functional Fas and also Fas ligand on thyroid follicular cells which can lead to self-apoptosis [41]. Furthermore, the accumulation of activated T cells expressing Fas ligand may induce apoptosis of thyrocytes directly by interacting with Fas on the cells [42].

The characteristic histopathological abnormalities of HT are profuse lymphocytic infiltration, lymphoid germinal centers, and destruction of thyroid follicles. Intrathyroidal lymphocytes are both T and B cells. Recent insight into the development of intrathyroidal germinal centers and lymph vessels suggests the importance of local production of chemokines [43]. B cells from thyroid tissue of patients with HT are activated, as indicated by their ability to secrete thyroid antibodies spontaneously *in vitro*. Thus, the thyroid gland may be a major site of thyroid Ab secretion. Anti-Tg and anti-TPO Ab of the appropriate IgG subclass have the potential to fix complement, and thus antibody-mediated complement-dependent cytotoxicity (CDC) may contribute to thyroid damage in some patients with HT [44]. More important may be the role of thyroid Ab secreting B cells in presenting thyroid antigen to the T cells, which react with processed thyroid antigens and peptides derived from these antigens. These activated T cells secrete cytokines, which themselves activate a variety of other immune cells. T cells have two roles in this disease: a role in Ab production (a Th2 type of function) and a role in the apoptotic destruction of thyroid cells by activating cytotoxic T cells (a Th1 function) [45]. The Th1 CD4⁺ lymphocytes, when stimulated by antigen, secrete interleukin-2 (IL-2), interferon gamma, and tumor necrosis factor-beta. In contrast, Th2 cells, when stimulated by antigens, secrete IL-4 and IL-5. Both types of T cells are found in thyroid tissue of patients with HT, but Th1 cells predominate [46, 47]. A T-cell clone that caused cytolysis of autologous thyroid cells has been reported in a patient with HT [48]. Finally, in addition to the various mechanisms of thyroid cell death, decreased thyroid hormone production in HT may be caused by TBAb which rather than causing thyroid cell death, bind to the TSHR and interfere with the ability of thyrocytes to respond to TSH. The relative contribution of all of these immunopathophysiological mechanisms in HT varies from patient to patient.

Bioassays for TSHR-Blocking Autoantibodies

Definition of blocking activity

Anti-TSHR-Ab that bind to the TSHR and neither activate the cyclic AMP pathway nor stimulate thyroid hormone synthesis, but rather

act as TSHR antagonists inhibiting the activation of signal transduction pathways, are defined as blocking anti-TSHR-Ab or TBAb [49, 50].

Historical methodology

Early bioassays for the detection of TBAb were based on the same cell-based systems used for TSAb, including thyroid plasma membranes, thyroid tissue slices, and eventually cultured thyroid cells [51–53]. In contrast to TSAb bioassays, TBAb bioassays detected the ability of patient sera to block various concentration of bovine TSH (bTSH) or TSAb-stimulated cAMP levels. Alternative TBAb assay systems were sought to address the limitations associated with using thyroid tissue or primary thyroid cells. A major advance in the field was the isolation of a cell line from normal rat thyroid (Fisher rat thyroid cell line, FRTL-5), which endogenously expressed the TSHR and was TSH-dependent for growth [54]. Importantly, FRTL-5 cells retained the main features of normal thyroid functions, such as hormone response, iodide uptake and thyroglobulin synthesis and could be maintained over the relatively long culture period which used by several investigators [33, 49, 54].

Further improvement in the detection of blocking activity was achieved with a luminescent bioassay for TBAb utilizing the CHO-K1 cell line (named lulu^{*}) stably transfected with the human TSHR and a luciferase gene under control of a promoter with a cAMP responsive element [24]. Using CHO lulu cells and a luminescent assay for TBAb, twelve samples, previously shown to contain TBAb by an established method quantifying cAMP by RIA, were also positive by the luciferase-based assay [24]. The specificity of this assay was excellent in that 20/20 patients with systemic lupus erythematosus, 13/14 with rheumatoid arthritis and 12/12 with multinodular goiter were negative for TBAb. Bioassays using such engineered CHO cells have the advantage of expressing the human TSHR and they require less cumbersome procedures for cell culture [55].

Another CHO cell line was generated for the measurement of TBAb that stably expressed a TSHR/LH-CG receptor construct that was reported to have a reduced response to most TSAb [56]. This Mc1 + 2 TSHR has amino acid (AA) residues 8 to 165 from the extracellular region of the TSHR substituted by the residues 10 to 166 of the LH-CG receptor. The CHO Mc1 + 2 cell line was responsive to TSH as measured by cAMP induction [57]. In a follow up study the CHO Mc1 + 2 cell line was compared with a wild type human TSHR CHO cell line, W25, for the measurement of TBAb [56]. A chimeric TSHR was also used in a coated tube assay for the detection of TBAb employing an adaptation of the TSH binding-inhibitory format [58]. This method utilized HEK-293 cells in which the wild-type TSHR of the TBII assay was exchanged for a chimeric receptor in which supposed TSAb epitopes (AA 8–165) were replaced by a TSH/Luteinizing hormone-choriongonadotropin (LH-CG) residues resulting in a chimeric receptor (chimera A) [57]. In studies using this assay sera from patients with GD and AITD were grouped according to their activity in a TBAb bioassay. At the decision threshold, the chimera assay had a sensitivity of 78% for TBAb with a specificity of 90.2%, but correlation with the bioassay results was only moderate ($r=0.46$). The technique was purported to allow the differentiation between sera containing only TBAb, both TBAb and TSAb, only TSAb, or sera without any bioactivity. Despite such efforts to map and remove stimulation-specific epitopes and incorporate chimer-

ic receptors into assays for exclusive detection of TBAb, it is not clear that these efforts have been successful.

Currently available TSHR-blocking antibody assays

A bioassay is commercially available in Japan (Yamasa, Corp., Choshi, Chiba, Japan) that utilizes porcine thyroid gland cells for the measurement of TBAb with a cAMP RIA [59, 60]. RSR Limited (Cardiff, UK) offers a research-use only service to measure TBAb. This assay uses CHO cells expressing the human TSHR and TBAb is detected by inhibition of porcine TSH-induced cAMP [61]. In another assay, autoantibodies inhibit the binding of M22 labeled with biotin to TSHR-coated enzyme-linked immunosorbent assay (ELISA) plate wells [62]. Recently, a bioassay for the TBAb measurement based on luciferase expression was described [23, 25]. The assay uses the same Mc4 cell line, that is used in the FDA-cleared TSAb bioassay [22]. The chimeric construct in the Mc4 cells amino acid (AA) residues 262–368 of the human TSHR are substituted with the AA residues 262–334 from the rat LH-CG receptor [25]. There is no cell line available expressing a modified TSHR that binds exclusively to TSAb or TBAb including the Mc4 TSHR which was originally purported to have TBAb epitopes removed. The TBAb bioassay using CHO Mc4 cells was shown to provide excellent analytical performance and a high level of reproducibility [25]. A schematic of the TBAb bioassay principle is shown in ► Fig. 1. Early in the development of this TBAb bioassay, CHO cells expressing either the chimeric human TSHR (Mc4) or the wild-type (wt) TSHR were compared side by side. Both CHO cell lines were induced with different con-

centrations of bTSH. The half maximal inhibitory concentration of K1-70 MAb in CHO Mc4 cells was more than five-fold lower compared to the CHO wt cells. The CHO Mc4 cell line showed several advantages over the CHO wt cell line including a broader linear range in response to bTSH, higher sensitivity in detecting blocking activity of the purely human blocking MAb K1-70 as well as detecting higher levels of blocking activity of TBAb positive sera from patients with AITD [23, 25].

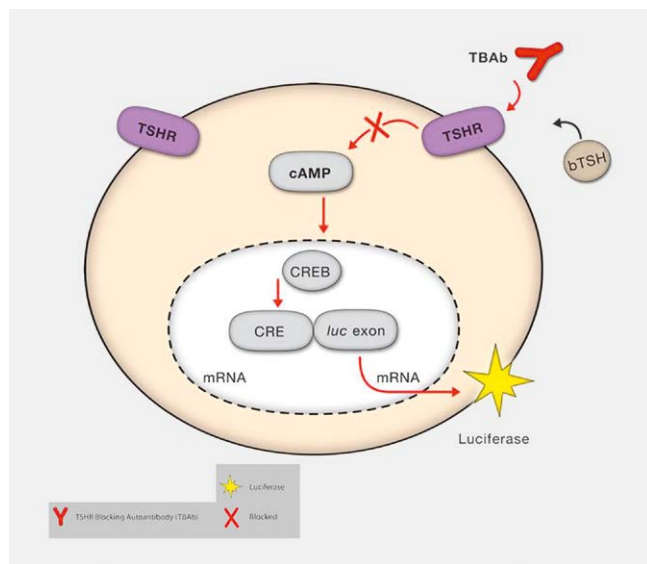
Another TSAb/TBAb bioassay was reported that utilizes a unique cAMP detection method. CHO cells were engineered to express the TSHR and a calcium-activated bioluminescent protein [63]. Following TSHR activation, increased intracellular cAMP levels activate the cyclic nucleotide-gated calcium channel. The subsequent influx of calcium results in bioluminescence of aequorin which is quantified with a luminometer [63]. As with other TSAb bioassays, TBAb can be measured with minor modifications of the protocol [63].

► Table 2 summarizes first generation (cAMP RIA) and second generation (cAMP/luciferase) bioassays described for the measurement of TBAb. Although head-to-head comparisons are limited, the variable methodologies used to detect TBAb do not give identical results and thus it is difficult to compare studies on the prevalence of TBAb [24]. Finally, it is important to note that when sera contain both TSAb and TBAb, bioassays measure the net activity of stimulating and blocking antibodies and dilutional analysis of serum may be required to detect both activities [13, 24]. At the time of publication there are no TBAb bioassays approved by U.S. or European regulatory agencies. Although second generation cAMP and luciferase assays using CHO cells have simplified the TBAb assay procedure, the availability of TBAb bioassays remains limited. Hopefully, this will change in the near future with increasing recognition of the importance of TBAb in evaluating and managing patients with AITD.

Clinical Relevance and Clinical Applications of TBAb

Studies on TBAb in primary hypothyroidism

One of the first studies that identified TBAb in patients with AITD reported on the inhibition of TSH-induced cAMP increase by IgG from patients with primary myxedema [29]. IgG fractions prepared from sera of patients with primary myxedema, goitrous HT, and controls, respectively, were tested for their ability to alter TSH stimulation of cAMP production in cultured human thyroid cells and the binding of TSH to its receptor. When compared with the cAMP increase induced by bTSH in the presence of normal IgG, cAMP accumulation was significantly inhibited by IgG from patients with primary myxedema. TSH-induced cAMP accumulation was not affected by IgG from patients with goitrous thyroiditis. These authors further studied TSH-stimulated cAMP response inhibitory immunoglobulins in patients with atrophic (primary myxedema) and goitrous HT and found TBAb in 40.5% and 17% patients with atrophic and goitrous thyroiditis, respectively [64]. In another study the prevalence of TBAb was also evaluated in hypothyroid patients with either goitrous or atrophic thyroiditis [30]. IgG was incubated with porcine thyroid cells in the presence of bTSH and the results were



► Fig. 1 Schematic of reporter gene-based functional bioassay for TBAb. CHO cells are engineered to constitutively express the human TSHR and to express luciferase following induction of cAMP. The binding of bovine (b) TSH to the TSH receptor on the surface of the cells induces a signaling cascade that leads to an increase of intracellular cAMP and subsequently to the luciferase expression. However, the presence of blocking anti-TSHR-Ab (TBAb) in serum of patients with autoimmune thyroid diseases inhibits the bTSH stimulation of the luciferase reporter gene. The TBAb level is correlated with blocking activity which is defined as percent inhibition of luciferase expression relative to induction with bTSH alone.

► **Table 2** First generation (cAMP RIA assays) and second generation (cAMP/luciferase assays) for TSHR blocking autoantibodies.

	Year	Species	Assay time	Comments	Reference
FIRST generation assays (cAMP radio-immunoassay)	1978	Human	4 days	Human thyroid plasma membranes, adenylyl cyclase activity	[51]
	1980	Human	4 days	Human thyroid plasma membranes, IgG preparation by column chromatography through DEAE-Sephadex	[52]
	1983	Human	4 days	Thyroid adenoma cells, IgG fractions preparation by DEAE-Sephadex column chromatography	[29]
	1985	Human	4 days	Human thyroid plasma membranes, IgG preparation by DEAE-cellulose column chromatography	[53]
	1987	Rat	4 days	FRTL-5 cells, IgG preparation by DEAE Sephadex	[33]
	1989	Rat	1–2 weeks	FRTL-5 cells, IgG preparation by polyethylene glycol (PEG) precipitation	[68]
	1989	Rat	1–2 weeks	FRTL-5 cells, IgG extraction by affinity chromatography on columns of protein A-Sepharose	[66]
	1990	Rat	1–2 weeks	FRTL-5 cells, IgG dialyzed in hypotonic buffer	[49]
	1999	Porcine	2 days	Porcine thyroid cells, IgG preparation by PEG precipitation	[59]
	1999	Hamster	2 days	CHO cells (clones JP02 and JP26)	[73]
	2000	Hamster	3 days	CHO cells (clone JP26)	[55]
SECOND generation assays (cAMP / luciferase)	1994	Hamster	4 days	CHO JP09 cells transfected with the recombinant human TSHR, IgG dialyzed and diluted in hypotonic buffer	[17]
	2001	Hamster	1 day	CHO cells (clone JP09) stably transfected with the TSHR (clone lulu *)	[24]
	2004, 2009	Hamster	20–24 h	CHO cells expressing the wild-type human TSHR, use of serum, spectrophotometric cAMP immunoassay	[62, 83]
	2013	Hamster	20 h	Fresh frozen vials of CHO cells expressing the chimeric human TSHR Mc4 and a luciferase reporter gene, use of serum, luminescence (cAMP-dependent luciferase expression)	[23, 25, 76]
	2015	Hamster	8 h	CHO cells expressing the wild-type human TSHR, use of serum, no sterile conditions necessary, luminescence (cyclic nucleotide-gated calcium channel and aequorin)	[63]

expressed as the percent inhibition of TSH-stimulated production of cAMP, as compared with production in a pool of IgG from normal subjects [30]. TBAbs were detected in 9% and 25% of the patients with goitrous and atrophic thyroiditis, respectively [30]. In a case report TBAbs were detected in a pre-pubertal severely hypothyroid boy (baseline serum TSH 254 mU/l) with atrophic HT and decreased growth velocity [65].

Potent blocking type anti-TSHR-Abs were observed in the majority of patients with primary myxedema using a FRTL-5 cell-based bioassay in which the prevalence of TBAbs in primary myxedema was 75% versus 0% in goitrous HT [66, 67]. In line with this, inhibition of TSH-stimulated RAI uptake in FRTL-5 cells by crude Ig fractions was reported for patients with goitrous and atrophic HT as well as from patients with overt hypothyroidism [16, 68]. TSH-stimulated RAI uptake was inhibited by the Ig fractions from TBII-positive patients with atrophic thyroiditis due to their TBAb positivity. The mean inhibition of TSH-stimulated RAI correlated with the ability of the Ig fractions to inhibit TBAb-stimulated RAI uptake ($r = 0.882$) and TSH-stimulated cAMP accumulation ($r = 0.929$) [16]. TBAbs were also present in 6/41 (14.6%) patients with hypothyroidism [68].

Other groups also evaluated Ab blocking the TSH-dependent cAMP production in FRTL-5 cells [33]. TBAbs were evaluated in patients with primary autoimmune hypothyroidism and were detect-

able in 15/23 patients with untreated idiopathic myxedema, and in 2/15 patients under L-T4 treatment. IgG from normal subjects or from 10 patients with non-autoimmune hypothyroidism did not cause any significant effect on the TSH-stimulated cAMP production. No correlation was found between TBAbs and the thyroid microsomal Ab. These authors further studied the incidence of Ab blocking the TSH effect in vitro in patients with euthyroid or hypothyroid HT [49]. TBAbs were detected in 12/26 (46%), 1/27 (3%), 3/32 (9.4%), and in 20/55 (36%) patients with atrophic thyroiditis, euthyroid HT, HT + subclinical hypothyroidism, and in hypothyroid HT, respectively. The prevalence of TBAbs was higher in atrophic thyroiditis vs. all other collectives. Mean TBAb levels in atrophic thyroiditis were higher than those in hypothyroid HT and subclinical HT. An inverse correlation was found between TBAb levels and estimated goiter weight.

The detection of Ab blocking TSH effects using CHO cells transfected with the cloned human TSHR was reported [17]. All IgGs producing an inhibition greater than two standard deviations from the mean of controls (> 25%) were considered positive for TBAbs. TBAbs were detected in 1/8 (12.5%), 7/30 (23.3%), and 16/47 (34%) patients with subclinical hypothyroid HT, hypothyroid HT and atrophic thyroiditis, respectively. This group used the same bioassay to demonstrate that humoral thyroid autoimmunity was not

involved in the pathogenesis of myxedematous endemic cretinism [69]. Further and unlike TSAb, which are restricted to the IgG1 subclass suggesting an oligoclonal origin, the IgG subclass distribution of TBAb in primary hypothyroidism are not restricted to a single subclass and are therefore likely to have a polyclonal origin [70].

Transient TBAb

The often transient nature of TBAb was noted in a report that described the disappearance of TBAb and the spontaneous recovery from hypothyroidism in a patient with autoimmune thyroiditis [32]. In another study, 21 TBAb-positive patients were followed for 6–11 years. TBAb disappeared in 15 patients (group 1), and persisted in six patients (group 2) [32]. Six patients in group 1 remained euthyroid, and nine became hypothyroid again within three months [32]. In contrast, all six patients with persistent TBAb positivity remained hypothyroid [32]. These authors, using a bioassay measuring porcine thyroid cell cAMP production, reported on their long-term follow-up (> 10 years) of TBAb-positive patients with hypothyroidism associated with atrophic and goitrous thyroiditis [30]. TBAb disappeared in 15/34 TBAb-positive patients with hypothyroidism. The disappearance of TBAb correlated with recovery from hypothyroidism in 13 (87%). All 10 TBAb-positive goitrous patients recovered from hypothyroidism while 19/24 (79%) TBAb-positive patients with atrophic thyroiditis continued to be hypothyroid. Two of the 34 TBAb-positive patients with hypothyroidism developed TSAb-positive Graves' hyperthyroidism. In another study changes in stimulating and blocking Ab were evaluated in a patient undergoing three cycles of transition from hypo- to hyperthyroidism and back to hypothyroidism [71]. Throughout the entire course of variation in thyroid function, TSAb were consistently present, but TBAb appeared and disappeared. Monitoring such activity indicated that the emergence of TBAb heralded the onset of hypothyroidism. A change in activity from TSAb to TBAb was also observed in GD patients during pregnancy and may have contributed to the remission of GD during pregnancy [72]. Median TSAb levels decreased during pregnancy while TBAb increased. The increase of TBAb was observed among those who were in clinical remission before pregnancy. A negative correlation was observed between TBAb activity and free T4 levels during pregnancy.

TBAb in GD

A number of investigators have evaluated the prevalence of TBAb in GD. One study used CHO cell lines expressing different TSHR numbers (JP09 and JP26) and found that TBAb were frequently detected in GD [73]. Thirty-four (40%) of 86 TSAb-negative GD patients were positive for TBAb. Differences between anti-TSHR-Ab bioactivity and inhibition of radioactive iodine (RAI)-labeled bTSH binding were noted in that only 78% of TBAb-positive sera detected with JP26 cells exhibited inhibition of RAI labeled bTSH binding [74]. Using JP09 CHO cells and unfractionated human serum, TBAb were detected in 4/24 (17%) TSAb-negative, TBII-positive hypothyroid GD patients [19]. Blocking type anti-TSHR-Ab were detected by a radio-receptor bioassay in nine of 30 GD sera (30%) using un-solubilized porcine TSHR and FRTL-5 cells [75]. In 1079 unselected, consecutive patients with AITD and 302 healthy controls, TBAb was found in 4.2% of patients with GD [76]. Twenty-eight hyperthyroid GD patients on methimazole were followed for 13 years [77]. Eight of 28 (29%) developed TBAb. Patients with TBAb responded well

initially to ATD and showed earlier normalization of the serum T4 level compared to patients without TBAb.

Transplacental transfer of TBAb

In the first report on TBAb in neonatal hypothyroidism, IgGs inhibiting the cAMP response to TSH in human thyroid membranes were demonstrated in three infants with transient neonatal hypothyroidism suggesting that transplacental transfer of TBAb caused the hypothyroidism in these infants [78]. Further, transient neonatal hypothyroidism was found in a daughter of a mother who was receiving treatment for primary hypothyroidism due to HT. IgG from maternal serum blocked TSH-stimulated cAMP response, and cAMP-stimulated iodine uptake and organification in cultured thyroid cells [31]. Maternal TBAb were detected in mothers of babies with either congenital [37] or neonatal [79] hypothyroidism. The levels of TBAb, based on the ability of patient serum to inhibit TSH stimulated 3H-cAMP production following incubation of FRTL-5 or CHO-JP09 cells with 3H-adenine, decreased to control levels in the child with neonatal hypothyroidism within two months of birth but remained elevated in the mother's serum. Also, an infant with severe neonatal hypothyroidism due to transplacental passage of TBAb was described [36]. TBAb were detected using a cell line, which stably expresses the human TSHR and a cAMP-responsive luciferase reporter by their ability to inhibit TSH-stimulated luciferase expression. High levels of TBAb were detected in maternal serum and initially in the infant's serum, but TBAb decreased in the infant to within the reference range by 3–4 months of age, illustrating the transient nature of this condition. Surprisingly, the thyroid function of this child did not return to normal on withdrawal of L-T4 therapy at 16 months of age when he developed transient compensated hypothyroidism.

Isolation and in vivo Effects of Human Blocking Monoclonal Antibodies

Human MAb against the TSHR were isolated from EBV-immortalized B-cells of patients with AITD both to understand the mechanism of the TSHR activation by Ab in patient serum and for the possible development of new treatments [40]. The serum of patients with AITD contains a mixture of polyclonal Ab whereas it was hypothesized that MAb, by virtue of their binding to a single epitope on the TSHR, could exhibit either pure stimulating or pure blocking activity in bioassays. This turned out to be the case and there have been several purely stimulatory and purely blocking MAb reported. Interestingly, a blocking MAb and a separate stimulating MAb were isolated from the same patient with GD [40].

Two human TSHR blocking MAb 129H8 and 122G3 were isolated from patients with GD that did not exhibit stimulatory activity in assays of thyroid function, but rather showed blocking activity in a bioassay [80]. A TSHR blocking MAb, 5C9 was also generated from the peripheral blood mononuclear cells of a female patient with post-partum thyroiditis and high serum TBII levels (260 U/l). This patient's serum inhibited the stimulating activity of TSH, a stimulating human MAb (M22), mouse TSHR stimulating MAb and TSAb from human serum. 5C9 IgG bound with high affinity (4×10^{10} l/mol) to the TSHR and inhibited the binding of TSH and M22 to the

receptor. Similar to the patient's serum, 5C9 IgG preparations inhibited the cAMP stimulating activities of TSH, M22, serum TSAb, and TSHR-stimulating mouse MAb [81].

The human TSHR blocking MAb K1-70 was isolated in 2010 [40]. B cells were obtained from the peripheral blood of a 54-year old woman with hypothyroidism and elevated TBII (160 U/l). The patient had an eight year history of AITD and she initially presented with hyperthyroidism, which was treated with methimazole. She then developed hypothyroidism and received levothyroxine. Evaluation of her serum showed both TSAb and TBAb with stimulating activity seen up to a dilution of 1/100 and blocking activity of 82% inhibition at 1/10 dilution. Lymphocytes were infected with EBV and fused with a mouse/human hybrid cell line (K6H6/B5). Using a TBII assay supernatants of twelve thousand hybridoma clones were screened to identify the K1-70 clone which stably expresses around 40 mg/l of IgG. K1-70 IgG was purified from culture supernatants using protein A affinity chromatography. K1-70 inhibited cAMP induction by TSH, a stimulating MAb, and TSAb-positive sera in TSHR-expressing CHO cells [40]. K1-70 IgG1 lambda showed high binding affinity (4×10^{10} l/mol) to the TSHR and bound to leucine-rich repeats 1–10 [82]. Furthermore, K1-70 was able to inhibit the stimulating activity of M22 in CHO cells expressing the human TSHR [82].

Human MAb produced against the TSHR were utilized to study their effects in vivo in laboratory animals [83, 84]. K1-70 was a potent inhibitor in vivo and was able to block the stimulating activity of M22. Furthermore K1-70 inhibited thyroid hormone secretion in rats following the intramuscular injection of 10–200 µg per animal [84]. In rats treated with K1-70 alone, the total T4 and free T4 decreased in a dose-dependent manner. These results demonstrate that the activity of these MAb observed in bioassays correlates with their in vivo activities. Further, it provides strong evidence that the bioassays in which the activity of these anti-TSHR MAb were measured, though contrived for convenient measurement, are nevertheless physiologically relevant.

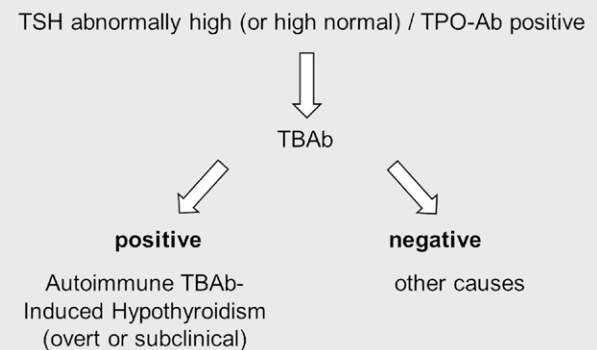
Clinical Indications for TBAb and Concluding Remarks

Our laboratory uses a TBAb bioassay based on the CHO/Mc4 luciferase cells used in an FDA-cleared TSAb assay. When evaluating serum samples of approximately 2000 unselected, consecutive patients with AITD, seen and followed at our institution, TBAb were prevalent in both subjects with HT (11%) as well as in those with GD (8%). The results of the TBAb measurement in the first 1400 patients have been reported [25, 76]. It is becoming clear that in addition to the well-known T-cell-mediated cytotoxicity and the involvement of CDC in HT, thyroid dysfunction and hypothyroidism can be induced by the occurrence and presence of TBAb. We and others have noted the occurrence of TBAb in patients with GD post radioactive iodine therapy and/or during treatment with antithyroid drugs [85], in patients with HT of recent onset, in AITD during pregnancy, as well as patients with non-thyroidal autoimmune disorders, that is, type 1 diabetes [38, 86–89]. The occurrence of TBAb during or after antithyroid drug treatment can impact the course of GD with TBAb-positive patients going into early remission or becoming spontaneously hypothyroid [85, 88]. Also, the majority of TBAb-positive subjects with

► Table 3 Potential indications for the measurement of thyrotropin receptor blocking antibodies.

1. Graves' disease: post radioactive iodine treatment and/or during therapy with antithyroid drugs
2. Autoimmune Hashimoto's thyroiditis
3. Autoimmune thyroid disease in pregnancy
4. Neonatal hypothyroidism
5. Postpartum thyroid disease
6. Differential diagnosis of thyroiditis
7. Differential diagnosis of hypothyroidism
8. Down Syndrome*
9. Non-thyroidal autoimmune diseases (SLE, RA, type 1 diabetes, myasthenia)

* According to reference [92]; SLE: Systemic lupus erythematosus, RA: Rheumatoid arthritis.



► Fig. 2 Diagnostic flowchart for TSHR blocking antibody-induced hypothyroidism.

HT were hypothyroid in contrast to TBAb-negative subjects who were predominantly euthyroid [76]. Regarding pregnancy and AITD, there is a real risk for the fetus in pregnant women with TSHR autoantibodies since both stimulating and blocking anti-TSHR-Ab can cross the placenta. Further, the risk for fetal/infant hyper- and hypothyroidism correlates with TSAb/TBAb levels [90, 91]. Therefore, and as listed in ► **Table 3**, there are several potential clinical indications for the measurement of TBAb in patients with AITD. Further, and as illustrated in ► **Fig. 2**, in patients with abnormally elevated basal serum TSH and suspected AITD, the measurement of TBAb can help diagnose humoral immunity-induced hypothyroidism in which thyroid dysfunction is caused by TBAb. However, and as previously acknowledged in the introduction, when compared to the abundant literature pertaining to the clinical applications of TSAb, more prospectively designed, controlled studies are warranted to gain more insight and daily experience regarding both the clinical utility of measuring TBAb as well as their potential implications for diagnosis, differential diagnosis, management and follow-up of patients with AITD.

Conflict of Interest

TD has nothing to disclose. PDO and GJK consult for Quidel, USA.

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