

Review

Thyroid Stimulating Immunoglobulin (TSI) in Graves' Disease

YUKIO OCHI, TAKEHIRO INUI*, TSUYOSHI KOUKI*, KEI YAMASHIRO*,
TAKASHI HACHIYA*, AND YOSHIHIRO KAJITA**

Emeritus professor, Shiga University of Medical Science, Shiga 520-2192,

**Department of Clinical Laboratory Medicine, Shiga University of Medical Science, Shiga 520-2192, and*

***Department of Internal Medicine, Nantan General Hospital, Kyoto 629-01, Japan*

IT HAS been 40 years since the discovery of long acting thyroid stimulator (LATS), now known as the thyroid stimulating antibody (TSAb) or thyroid stimulating immunoglobulin (TSI). An immunoglobulin G (IgG) with thyroid stimulating activity accepted as the etiological factor of hyperthyroidism in Graves' disease. On the other hand, IgG which inhibits TSH binding to and stimulation of the TSH receptor is called thyroid stimulation blocking antibody (TSBAb). These 2 types of IgG are considered to be TSH receptor antibodies (TRAb) and are currently measured by the TSH receptor assay and bioassay. In the TSH receptor assay, IgG which inhibits TSH binding to the receptor is called TSH binding inhibitory immunoglobulin (TBII). TSAb and TSBAb are determined by measuring cAMP (marker of thyroid stimulation) produced in thyroid cells exposed to IgG in the absence and presence of TSH, respectively [1, 2].

Since clarification of the structure of the TSH receptor the binding epitope for TRAb has been studied, but the nature of the epitope for TRAb is still controversial. We review the recent advance in knowledge concerning TSI and TRAb research and describe recent results obtained in our laboratory.

LATS

LATS was the first good example of an antibody capable of stimulating specific cell function. LATS may stimulate the thyroid of mammalian species such as human [3, 4], bovine [5], porcine [6], rat [7], guinea-pig [8] and mouse [9], but does not stimulate the non-mammalian thyroid such as in birds and amphibia [10, 11]. On the other hand, TSAb (without LATS activity) may stimulate the human, bovine and porcine thyroid but not guinea-pig or mouse. TSH (mammalian species) stimulates the thyroid of all animal species [10, 11]. It is suggested that LATS may have thyroid stimulating (TS) activity for lower developed animals (rodents such as guinea-pig, rat and mouse) compared with TSAb (without LATS activity) [11] (Table 1). When TBII (with TSH receptor assay) and TSAb [cAMP production in porcine thyroid cells (PTC) assay] were determined, all LATS positive sera showed extremely high TSAb and TBII activities [12]. All available data indicate that TSH and TSAb may bind to the same site on the TSH receptor and that TSAb may be TRAb with a thyroid stimulating action [1, 2].

Anti-TSH Antibody in Graves' Patients

Abnormally negative values in the TRAb (TBII) assay led to the discovery of bTSH binding antibody. The existence of anti-bTSH antibodies has been reported mainly in Graves' disease (0.1–0.3 % of Graves' disease) but also in other thyroidal diseases (Hashimoto' thyroiditis, silent thyroiditis

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Correspondence to: Dr. Yukio OCHI, Research Institute for Production Development, 15 Morimoto-cho, Shimogamo, Sakyo-ku, Kyoto 606-0805, Japan

Table 1. Comparison of thyroid stimulatory actions of LATS positive and LATS negative TSAb

Species	Thyroid	Thyroid stimulation		
		TSAb		TSH
		LATS (+)	LATS (-)	
Mammalian	Human	+	+	+
	Monkey	+	+	+
	Dog	+	+	+
	Bovine	+	+	+
	Porcine	+	+	+
	Rat	+	+	+
	Guinea-pig	+	-	+
	Mouse	+	-	+
Bird	Chick	-	-	+
Amphibia	Tadpole	-	-	+

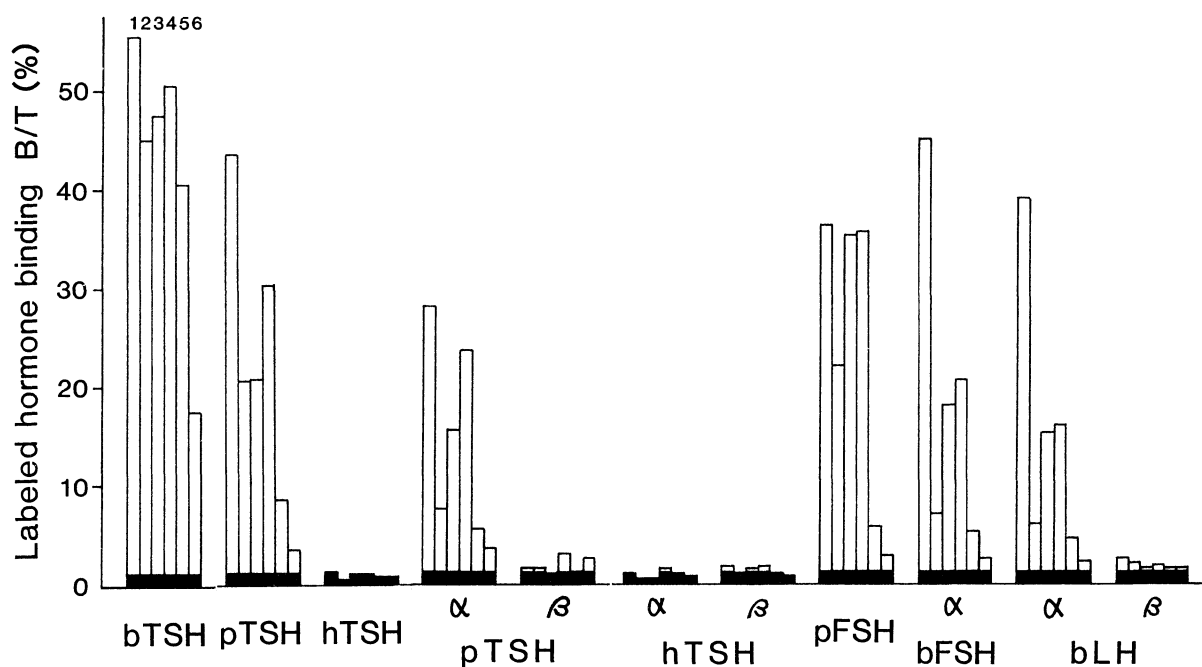
TSAb, thyroid stimulating antibody; LATS, long acting thyroid stimulator.

and subacute thyroiditis). These TSH binding antibodies showed a reaction with TSH in several mammalian species except humans [13]. We demonstrated that almost all TSH binding antibodies reacted not only with bovine and porcine TSH, FSH and LH, but also with the α -subunit of these 3 hormones, but no reactivity was observed

with the β -subunit of these 3 hormones. These results indicate that these TSH antibodies mainly recognize epitopes in the α -subunits of bovine and porcine pituitary glycoprotein hormones (TSH, FSH, LH) (Fig. 1). The existence of this kind of antibody suggests the involvement of an autoimmune mechanism for the production of these antibodies not only in the thyroid but also in the pituitary. It is therefore better to refer to this subgroup of Graves' disease as the pituitary glycoprotein hormone α -subunit antibody syndrome. The antigen responsible for production of anti-TSH antibodies and also the pathological significance of these antibodies are unclear.

Some reports have suggested that the TRAb in Graves' disease may be an anti-idiotypic antibody to anti-TSH antibody. This hypothesis is attractive, but binding of labeled bovine (b)TSH or porcine (p) TSH to these bTSH antibodies was not inhibited by TRAb in Graves' disease [14, 15]. Therefore, there is as yet no evidence to support the hypothesis that anti-bTSH antibodies are involved in an anti-idiotypic antibody network in Graves' disease.

In the standard TBII assay, the TBII activity in test serum is calculated by the nonspecific bTSH binding to normal pool serum [NSB (N)] without

**Fig. 1.** Binding of various hormones and their subunits with TSH antibody in Graves' disease. The black area indicates the normal range.

determining NSB in test serum [NSB (T)]. When the standard TBII activity in patients was abnormally negative, the corrected TRAb activity [calculated by determining NSB (T)] in these cases showed highly positive TBII activities. But the corrected TBII activity did not indicate genuine TBII activity because of the extremely high NSB (T) values compared to those of NSB (N). We reported a method for the precise determination of TBII activity in serum containing bTSH antibody by complete absorption with heat-denatured bTSH or sheep FSH [16].

TSH Binding Inhibitory Protein (TBIP) and Calmodulin (CaM)

CaM is known as a general regulator of cell function. Several studies have shown that CaM may play a role in cell proliferation. CaM was reported to play a role in the activation of human thyroid adenylate cyclase by TSH. This adenylate cyclase is one of several enzymes known to be activated by CaM.

We previously demonstrated that authentic CaM (bovine brain) had TSH binding inhibitory (TBI) activity on TSH receptors in the human and porcine thyroid and on the guinea-pig epididymal fat membrane. The TBI activity of authentic CaM in the TSH receptor assay with recombinant hTSH receptor was abolished by EGTA [17, 18]. Recently we found that the TBIPs purified from human and porcine thyroids were, in fact, CaM. The effects of authentic CaM and the TBIP purified from the human thyroid on cAMP production stimulated by TSH or TSAb in PTC were examined. Neither authentic CaM nor TBIP itself increased basal levels of cAMP production, but inhibited cAMP production stimulated by TSH. Authentic CaM and TBIP showed no sign of an inhibitory action on cAMP production stimulated by TSAb or that induced by various thyroid stimulators [GTP γ S, forskolin and pituitary adenylate cyclase-activating polypeptide (PACAP, 27 and 38 amino acids)]. These results suggest that TSH and TSAb have different binding sites on the TSH receptor because of different effects of CaM on thyroid stimulation by TSH and TSAb, although it is still unclear whether TSAb binds to the same receptor as TSH [19]. If TSH and TSAb have similar binding sites

which are linked to thyroid stimulation and different binding sites which are linked to non-thyroid stimulation, the latter is suggested as a binding site of CaM. CaM may have an inhibitory effect on not only TSH binding to thyroid membranes in the TSH receptor but also on TSH stimulation of intact thyroid cells in the bioassay method. As far as I know, there have been no studies of the effect of CaM on hormone responsiveness. Determination of the sequence of the CaM domain that binds to the receptor and prevents TSH interaction may facilitate development of therapeutic methods to block the TSH receptor by expression of this sequence [20].

Discordance between TBII and TSAb Activity

TBII does not represent TSAb activity even in untreated Graves' disease. There were several cases in which TSBAb positive hypothyroidism changed to TSAb positive hyperthyroidism [21]. Cases with the co-existence of TSAb and TSBAb [22] and cases in which TSAb activity changed to TSBAb activity after radioisotope treatment in Graves' disease have been reported [23]. These lines of evidence suggest that the biological activity of patient's IgG depends on the differences in amount of TSAb and TSBAb, so that TBII and TS activities in the co-existence of TSAb and TSBAb may be expressed by the following formula.

$$\text{TBII activity} = \text{TBII activity derived from TSAb} + \text{TBII activity derived from TSBAb}$$

$$\text{TS activity} = \text{TSAb activity} - \text{TSBAb activity}$$

Co-existence of both TSAb and TSBAb may lead to difficulties in interpreting assay data, but the precise determination of TBAb, TSBAb and TBII activity may resolve such discordance between TSAb and TBII in the near future.

Fragments of TSAb-IgG with Thyroid Stimulating (TS) Activity

Previous studies have shown that LATS and TSAb behave like an antibody in enzymatic digestion because the TS activity is distributed in both F(ab')₂ (divalent) and Fab fragments (monovalent) of IgG [24]. Recently, we re-examined TS active portions in LATS and TSAb molecules.

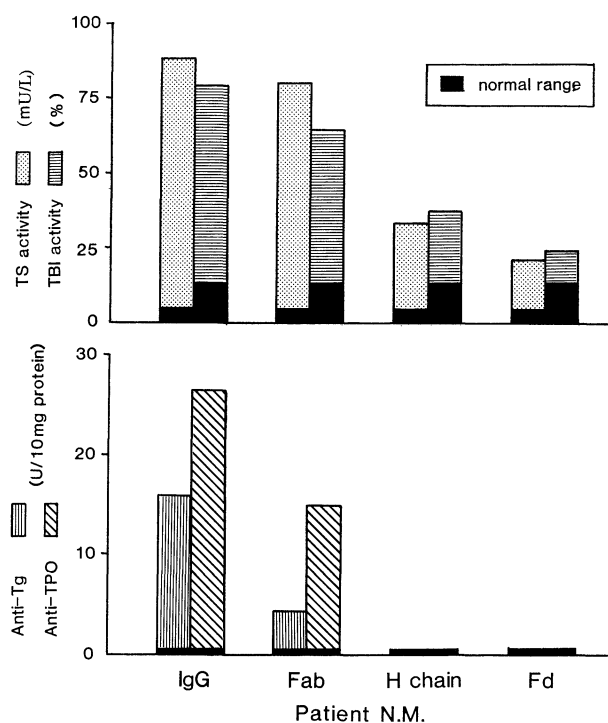


Fig. 2. Thyroid stimulating activity in H chain and Fd fragment of TSAb-IgG produced by papain digestion and reduction. TS activity (mU/L) indicates bTSH equivalent. The black area indicates the normal range.

Biologically active fragments of LATS and TSAb-IgG molecules after proteolytic digestion were examined. Both TS activity (cAMP production in PTC) and TSH binding inhibitory (TBI) activity (determined by TSH receptor assay) were found in not only the Fab fragment but also in a smaller molecular component (20 KDa) which we tentatively designated as thyroactive smaller components (TSC). We suggested that TSC may be released from the Fab fragment region of TSAb-IgG by proteolytic hydrolysis [25]. Furthermore, the free H chain fraction obtained by reduction of TSAb-IgG showed both TS activity and TBI activity, but the free H chain fraction showed neither thyroglobulin nor thyroid peroxidase (TPO) antibody activity. On the other hand, the free light (L) chain was not biologically active. Similar TS and TBI activities were found not only in IgG(κ) and IgG(λ) but also in the Fab(κ) and Fab(λ) fractions. The Fd fragment obtained by protease digestion and reduction also had both biological

activities. These results indicated that TSAb was polyclonal and that the TS activity was distributed within H chain fragments of TSAb-IgG [26] (Fig. 2).

TS Activity in TSBAb-IgG

TSBAb has been suggested to be the causal factor of hypothyroidism and is usually measured by inhibition of TSH-stimulated cAMP response. TSBAb is now considered to be blocking type TBII, but the epitope for TSBAb has not yet been well defined. The blocking action of TSH stimulation by TSBAb has been suggested to be due to its direct binding of the innocent IgG to the TSH receptor. On the other hand, TSAb (the stimulatory type TBII) has been demonstrated to bind to the TSH receptor and to stimulate cAMP production, so that innocent type TRAb (ITRAb) is a more suitable name than blocking type TRAb (TSBAb).

The genetical similarity of Graves' patients to TSBAb positive hypothyroid patients characterized by a decrease in HLA-DW2 in Japanese patients has been reported [27]. From the genetically intimate relationship between these two groups it has been suggested that they may belong to autoimmune TSH receptor disease [2].

The existence of TSBAb activity in F(ab')₂ and Fab fragments has been reported previously [28]. On the other hand, TSBAb has been suggested to show intrinsic TS activity [29, 30]. Recently, we found the appearance of TS activity after papain hydrolysis in 5 out of 7 TSBAb-IgGs (Fig. 3). The biologically active fragments with TS activity [Fab fragments and the smaller molecular components (Mr 20 KDa) similar to papain hydrolysis of TSAb-IgG] were found following papain hydrolysis of TSBAb-IgG. The conversion of TSBAb-IgG activity to TS activity by papain digestion suggests that the inherent TS activity located in the Fab portion of the IgG molecule is disclosed by papain digestion. Our results suggested that the TS activity in the smaller molecular component (Mr 20 KDa) may be released by hydrolysis of the Fab portion of the IgG molecule [31].

There are various mechanisms to explain the appearance of TS activity in TSBAb-IgG caused by papain digestion. One is the co-existence of both TSBAb and TSAb. Another possibility is derived

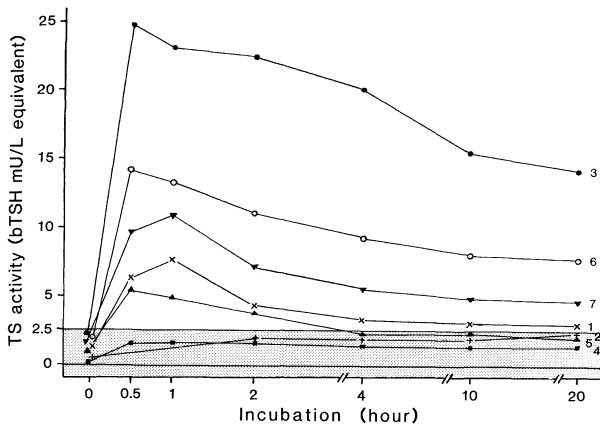


Fig. 3. Thyroid stimulating activity of papain digested TSBAb-IgG. The shaded area indicates the normal range.

from the disclosure of an intrinsic TS activity in TSBAb-IgG molecule by papain digestion. Subtle differences in binding characteristics, which are probably responsible for the functional difference between stimulation and blocking, may be important for the biological response.

Stimulation of PEG on cAMP Production by TSAb in Porcine Thyroid Cell (PTC) Assay

cAMP production by means of the polyethylene glycol (PEG, No 6000) 12.5% precipitated fraction (PF) from test serum in PTC assay has been performed in the routine TSAb assay method. cAMP production was examined with the PF formed by 12.5%, 15%, 17.5%, 20%, 22.5% and 25% PEG solutions. A gradual increase in cAMP production by PEG PF from Graves' sera was observed with increasing PEG concentrations, whereas no increase in the cAMP level occurred when using sera from normal subjects. The cAMP production by means of the PF with 22.5% PEG significantly exceeded that with 12.5% PEG in almost all Graves' sera examined in the present study [32] (Fig. 4).

Co-incubation of 5% PEG with TSAb-IgG (purified by the Protein A method) induced a maximum increase in cAMP production (approximately a 10-fold increase), whereas no increasing effect by PEG on TSH activity was observed. A stimulatory effect of PEG on TS

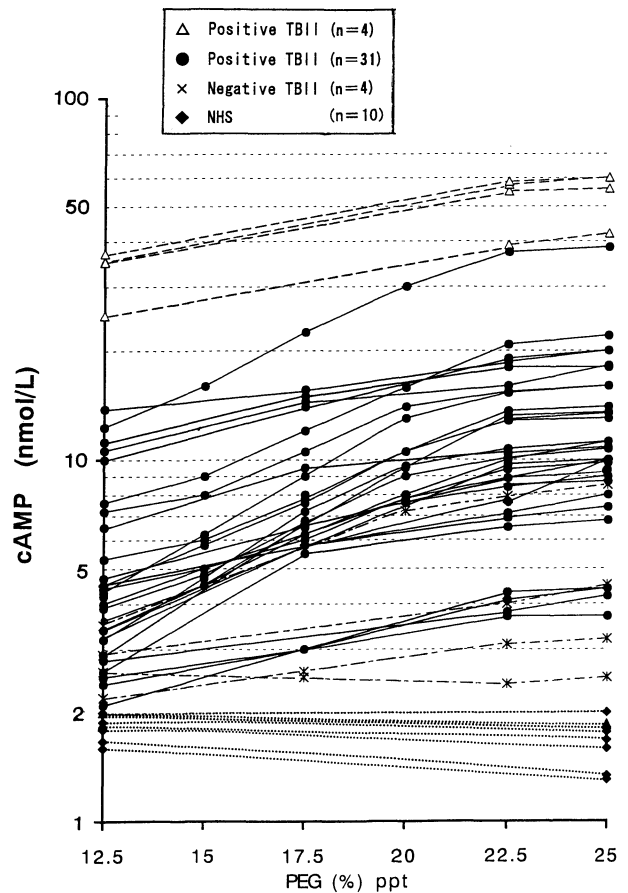


Fig. 4. cAMP production by PEG precipitated fractions from Graves' serum. All values are shown as the means of duplicate determinations.

activity by TSAb-IgG in PTC assays was observed without changes in TBII activity. On the other hand, no appearance of TS activity from TSBAb-IgG was observed following the addition of 5% PEG. No stimulatory effect of 5% PEG was observed with various thyroid stimulators such as GTP γ S, forskolin and pituitary adenylate-cyclase activating polypeptide (PACAP) (27 and 38 amino acids). A stimulatory effect of 5% PEG on TS activity produced directly by small amounts of Graves' serum ($\leq 50 \mu\text{L}$) was also observed.

The TS activity expressed by equivalent amounts of bTSH was more accurate than the TS activity expressed by the cAMP level shown as the standard curve of cAMP production by bTSH in the PTC assay. The increase in TS activity expressed by equivalent amounts of bTSH was higher than that expressed by cAMP amounts when the increase in

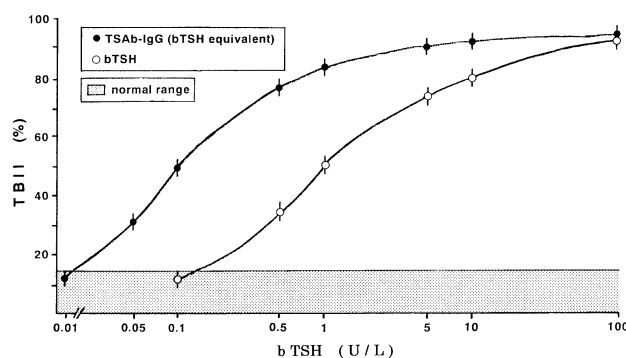


Fig. 5. TBII activity and thyroid stimulating activity (expressed by bTSH equivalent) in TSAb-IgG (purified from untreated Graves' serum by the Protein A method) and bTSH. Values are means \pm SD. The shaded area indicates the normal range.

TS activity caused by 5% PEG was compared.

Sera from untreated hyperthyroid Graves' patients show about 10 fold higher TBII activity than TS activity when both activities are shown as bTSH equivalent values. A typical case is shown in Fig. 5. TSAb activity in Graves' IgG with 50% TBII activity was 0.1 U/L as bTSH equivalent. On the other hand, bTSH preparation with 50% TBII activity showed 1 U/L of TS activity. The 10 fold increase in TS activity in TSAb-IgG with no change in TBII activity caused by the co-incubation of TSAb-IgG and 5% PEG in PTC assay may explain the differences in TSAb and TBII activities in reported Graves' IgG. The mechanism by which high PEG concentrations increase TS activity stimulated by TSAb-IgG without changes in TSH receptor binding (TBII activity) is unclear.

PEG has been used to promote cell fusion of both plant and animal cells. It is known that PEG causes cell shrinking and aggregation, decreases in membrane fluidity and also osmotic swelling [33]. PEG has also been used to accelerate the DNA ligase reaction [34].

One possibility is that cAMP production may be increased by TSH receptor binding resulting from the conformational change in TSAb-IgG due to PEG conjugation in PTC. Another possibility is the existence of some factors influencing this potentiality.

The TS activity caused by the PEG 12.5% PF from Graves' sera has been suggested to represent the

active form of TSAb-IgG, and the TS activity caused by the PEG 22.5% PF from Graves' sera is taken to indicate the total TSAb activity. The active form of TSAb-IgG usually accounts for 10–30% of the total activity in many Graves' sera.

Increase in TS Activity in TSAb-IgG Induced by Polyvinyl Alcohol (PVA) and Dextran

Recently we found the stimulatory effects of non-ionic hydrophilic polymers such as PEG, PVA and dextran which have been shown to promote cell fusion on cAMP production by TSAb in PTC assay. PVA and dextran increased TS activity of Graves' IgG similarly to PEG in the PTC assay. Co-incubation of 10% PVA or dextran T-70 showed significantly high cAMP production by TSAb. cAMP production by 10% PVA showed higher TS activity compared to 10 % dextran T-70 or 5% PEG (6,000) [35].

Clinical Usefulness of Highly Sensitive TSAb Assay

These experimental results indicate that the phenomenon of increased TS activity caused by high PEG concentrations in PTC assay is specific for TSAb, and that this PEG method is available for use as a highly sensitive TSAb assay which will be a useful indicator for the diagnosis of Graves' disease and also as a reliable monitor of the clinical course of Graves' patients. Precise TSAb determination may contribute to understanding the pathophysiology of Graves' disease. Further studies with thyroid cells to clarify the mechanism involved in the stimulatory effect on cAMP production by Graves' IgG are also needed.

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