

Differences in TSH Receptor Binding and Thyroid-stimulating Properties between TSH and Graves' IgG. Slowly-acting TSH Receptor Antibody Moieties in Graves' Sera Affect Assay Data

YASUHIKO SAIKI, TAKASHI ISHIHARA*, KATSUJI IKEKUBO AND TORU MORI**

Department of Nuclear Medicine, Kobe City General Hospital, Kobe 650-0046, Japan

*Department of Endocrinology, Kobe City General Hospital, Kobe 650-0046, Japan

**Department of Internal Medicine, Kyoto Senbai Hospital, Kyoto 605-0932, Japan

Abstract. We analyzed TSH receptor (TSHR) effects, both binding and thyroid-stimulation, of TSH and Graves' IgG. A new TRAb assay system utilizes rhTSHR coated tubes and is comprised of two step incubation, the first incubation with patient serum followed by a second incubation with ^{125}I -bTSH. We called TRAb measured by this method as hTRAb. ^{125}I -bTSH binding capacity of the tube was found close to saturation at 1 hr with 200 μl of ^{125}I -bTSH. Up to 5 hr of first incubation for hTRAb assay revealed significant increases in all hTRAb activities. hTRAb was not affected by second incubation time or dose of ^{125}I -bTSH. When 1 step incubation with ^{125}I -bTSH and Graves' serum was performed, hTRAb again increased significantly with time. A simple competitive equilibrium model could not be applied to these ligands. Second, Graves' IgG and bTSH were compared for *in vitro* thyroid-stimulation sequentially up to 24 hr, measuring cAMP generation from cultured porcine thyrocytes. While bTSH yielded peak cAMP generation by 8 hr, TSAb revealed more cAMP generation by 24 hr than at 8 hr. We concluded that individual Graves' sera contain heterogeneous TRAb of variable avidities, and that slow-acting TRAb, which may lack biological activity, can be detected by prolonged incubation.

Key words: TSH receptor, TSH, TSH receptor antibody (TRAb), Thyrotropin-binding inhibitor immunoglobulin (TBII), Thyroid stimulating antibody (TSAb)

(Endocrine Journal 52: 45–55, 2005)

ALMOST 50 years have passed since the discovery of abnormal thyroid stimulator in Graves' sera by Adams and Purves [1]. In view of the prolonged thyroid stimulatory activity of Graves' sera compared to TSH, the serum component was named Long-acting Thyroid Stimulator (LATS) [2]. Subsequently, the active substance(s) was found in the gamma-globulin fraction and assumed to be an autoantibody against certain antigen present in the thyroid tissue [3]. The antigen was further located to the TSH receptor (TSHR), and *in vitro* assay systems of TSHR antibody (TRAb) were developed for both receptor-binding and thyroid-stimulating

activities [4–6]. After the cloning of TSHR cDNA [7, 8], numerous efforts have been made to elucidate the binding sites of TSH as well as TRAb [9–15]. However, little has been known until now about the binding mechanisms of TSH and TRAb to TSHR [16]. Additionally, the presence of heterogeneity among Graves' TRAb has also been indicated [9, 17–20].

Recently, a sensitive TRAb assay method was established [21, 22]. This assay utilizes recombinant human TSHR (rhTSHR) coated tubes and is comprised of a two step incubation system. First, the serum component is subjected to receptor binding, and after washing labeled TSH is applied for binding. Through analyses using this system, we have compared the TSHR binding properties of TSH and Graves' serum component. Further, time-sequence effects of TSH and Graves' IgG on *in vitro* thyroid stimulation have also been compared.

Received: May 7, 2004

Accepted: October 12, 2004

Correspondence to: Dr. Takashi ISHIHARA, Department of Endocrinology, Kobe City General Hospital, Minatojima-nakamachi 4-6, Chuoku, Kobe 650-0046, Japan

Materials and Methods

1. TSH receptor binding studies

1) Conventional thyrotropin-binding inhibitor immunoglobulin (TBII) assay

We routinely measured TBII activity using a TRAb 2 kit, Cosmic Co. Ltd., Tokyo, Japan, which utilizes solubilized porcine thyroid cell membrane. TBII activity was expressed as % inhibition of ^{125}I -bTSH binding by previously added and then co-incubated patient serum. Nonspecific binding was obtained by the blank tubes provided which lack TSHR. % inhibition of each samples were calculated from $\{1 - (\text{bound counts by sample} - \text{nonspecific bound count}) / (\text{maximal bound counts} - \text{nonspecific bound count})\} \times 100$, while the maximal binding was obtained by application of a negative control serum provided. Normal range in our laboratory is <10%. Time sequence study extending the first incubation time with patient's serum from regular 15 min up to 120 min was also performed.

2) Newly developed sensitive TRAb assay procedures

A sensitive TRAb assay kit (DYNO test TRAb human) provided by Yamasa Corp., Tokyo, Japan was used throughout [21, 22].

The regular assay procedures are briefly as follows:

First incubation: 100 μl serum sample with 200 μl of incubation medium containing anti-hTSH antibody was incubated in rhTSHR-coated tube for 2 hours at room temperature. Second incubation: After thorough washing of the tube, ^{125}I -bTSH solution (200 μl) was added and incubated for 1 hour at room temperature. After washing, radioactivity in the tube was counted by a gamma-counter.

hTRAb activities were calculated as:

$(\text{Cpm by negative control} - \text{Cpm by sample}) / (\text{Cpm by negative control} - \text{Cpm by positive control}) \times 100\%$

Thus, this assay is essentially similar to TBII assay, but we designated the measured activities as hTRAb in order to distinguish it from those by TBII assay.

Negative control was prepared from a normal human serum pool, and was defined to contain 0 IU/ml of TBII. Positive control was prepared from a serum pool obtained from Graves' patients with potent TBII, and was adjusted to contain 40 IU/ml of TBII.

In our laboratory hTRAb higher than 15% was taken as positive, which was derived from ROC analysis of assay data obtained from 47 untreated hyperthyroid

Graves' patients and 100 healthy control subjects [23].

The company recommended use of IU/L expression; however, in this study we variably modified the incubation conditions and instead we took % inhibition, which we considered to be much more suitable for comparing experimental results with each other.

3) Modified assay procedures

In this study, the incubation conditions were varied. Times for the first incubation were studied at 30 min, 1, 2, 5 hours, and overnight, respectively. Serum volume applied was reduced to 50 μl as well. For the second incubation, various time courses of 30 min, 1, 2, and 5 hours were chosen. Applied ^{125}I -bTSH volumes were also changed to 100, 200, and 400 μl , respectively.

A 1 step incubation assay was also performed. Fifty μl aliquots of serum sample with the first incubation medium (200 μl) were mixed together with ^{125}I -bTSH solution (200 μl) and incubated for 1, 3, and 5 hours (total volume 450 μl). Radioactivities were counted just after washing.

2. Assays for thyroid-stimulating activities

Thyroid-stimulating antibody (TSAb) activities were measured using cultured porcine thyrocytes [24]. IgG fractions were prepared from a normal human serum pool and 3 sera from active Graves' patients. Onto 0.5 ml aliquots of serum, 1.5 ml of 18% PEG (6000) aqueous solution was added. After mixing, centrifugation and decantation, the precipitate was dissolved in 0.5 ml of salt-free Hanks' medium. Routinely, 200 μl of IgG solution was poured into each of 2 wells containing 5×10^5 cultured porcine thyrocytes, and incubated for 4 hours at 37°C under 5% CO_2 condition. Incubation was terminated by the addition of 10 μl 250 mM EDTA. After incubation, 25 μl aliquots of the incubated situation were aspirated and subjected to cAMP measurements by RIA. TSAb activity was calculated as cyclic AMP generated by sample IgG/ cAMP generated by normal IgG $\times 100$ (%), and values exceeding 180% were regarded as positive.

A purified bTSH preparation (Sigma, NY, USA) was dissolved by normal human IgG solution and diluted further to make the final TSH concentrations of 200 and 400 $\mu\text{IU/ml}$.

To see the time sequence effects of bTSH and Graves' IgG, 2, 4, 8 and 24 hour-incubations were performed in duplicate. After incubation, the aliquots

were aspirated, kept in storage below 4°C, and then subjected to cAMP measurements after being warmed to room temperature. These TSAb assays were kindly performed by Kazuyoshi Togashi at Yamasa Corporation.

3. Serum samples used and statistical analysis

Normal human serum samples were collected from employees of the Kobe City General Hospital known not to have any thyroid abnormality or any relatives with thyroid disorders and to be healthy in general check-ups. Graves' sera were obtained from patients with definitive diagnosis after obtaining informed consent individually.

All experimental results were presented as mean \pm SD. Student's paired *t* test was applied for within group comparisons. *P* values <0.05 were taken as significant.

Results

1. Comparisons of hTRAb assay results with those by conventional TBII assay

In order to evaluate the sensitivity and specificity of the new assay, 17 Graves' sera with various hTRAb and TBII activities were chosen. As shown in Fig. 1a, all 6 hTRAb and TBII positive samples showed significantly higher percent inhibition of 125 I-bTSH binding by the new hTRAb assay than TBII activities (*p* = 0.003). When serum volume applied was reduced to 50 μ l instead of 100 μ l, the hTRAb results obtained were between those by regular assay and conventional TBII assay. When incubation time was extended overnight, all samples gave further and significantly increased hTRAb results (*p* = 0.024). Fig. 1b shows results of 6 Graves' sera with positive hTRAb but negative TBII. This hTRAb assay was shown not only to increase percent inhibition of 125 I-bTSH binding but to be able to detect antibody activities which were undetectable by TBII assay. Results of 50 μ l assays also revealed positive hTRAb in all 6 sera tested. Further,

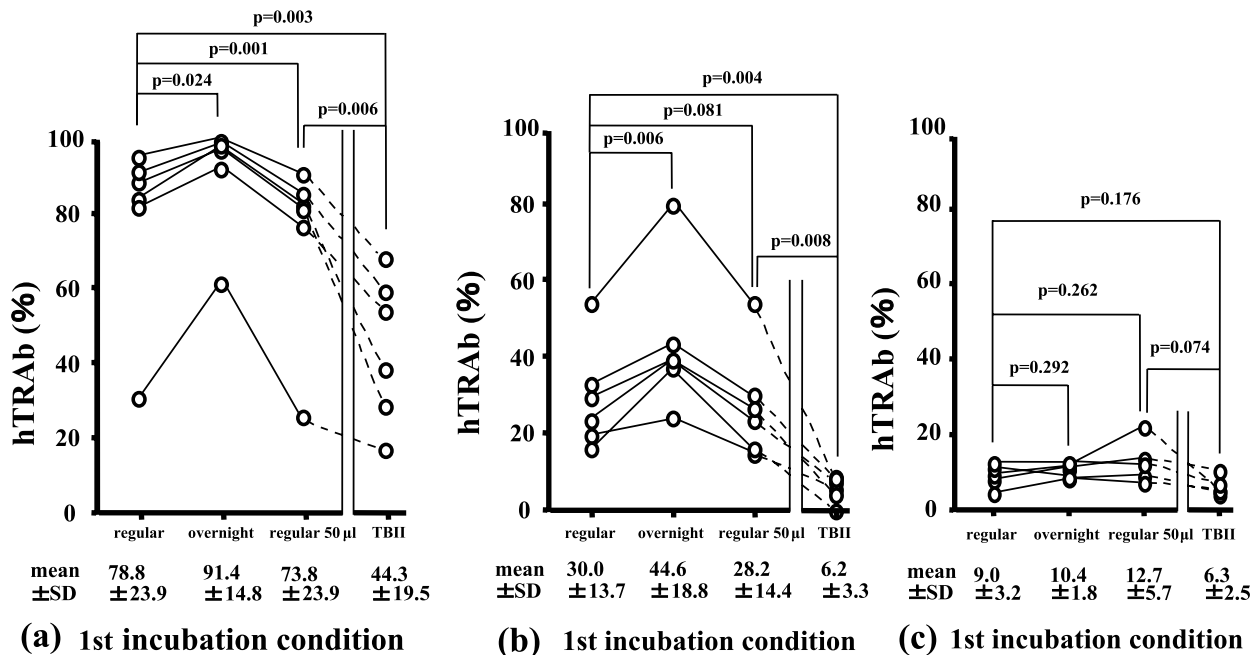


Fig. 1. Results of hTRAb assay by the new method.

Regular 2-hr first incubation hTRAb assay results with the new method were compared with those for overnight first incubation, reduced dose (50 μ l) assay, and conventional TBII assay results. Conventional assay was performed separately. Mean \pm SD of each points are presented on the bottom, while Student's paired *t* tests were used for statistical analyses. (a): Results of 6 sera with positive both hTRAb and TBII. (b): Results of 6 hTRAb positive but TBII negative sera. (c): Results of 5 hTRAb and TBII negative sera.

overnight incubation assay results showed much greater hTRAb increases than those with regular 100 μ l assay ($p = 0.006$). On the other hand, 5 sera with negative hTRAb and TBII samples remained negative throughout except one with 50 μ l assay (Fig. 1c). Overnight incubation did not show any hTRAb increases in these samples either. From these results this assay was found to be at least more than twice as sensitive as the conventional TBII assay and even able to detect low-titer antibodies newly, especially by prolonged incubation.

2. Binding capacity of the rhTSHR to TSH

Fig. 2a shows bound 125 I-bTSH counts (cpm) to the rhTSHR coated tubes after the regular first incubation for 2 hours using negative and positive control substances. Gradual increase in total bound cpm by negative control was observed according to the second incubation time, but non-specific binding obtained by positive control also increased, and calculated speci-

fically bound % showed elevation by 1 hour and not much further elevation thereafter. Fig. 2b shows 125 I-bTSH binding by variable doses applied after first incubations with negative or positive controls. Much increased binding was observed by 200 μ l applications than that of 100 μ l. However, no further increase was seen by 400 μ l dose and even a great decrease in specifically bound % was noted. These data indicate that TSH binding capacity of the rhTSHR is close to saturation by 1 hour incubation with regular 200 μ l dose of 125 I-bTSH applied.

3. Displacing effect of 125 I-bTSH on previously bound hTRAb

Fig. 3a shows alterations of hTRAb activities in 8 Graves' sera measured after 2 hour first incubation followed by various lengths of second incubation with 125 I-bTSH. To see the displacing effect of 125 I-bTSH more clearly, applied serum volumes were reduced to 50 μ l instead of regular 100 μ l. Unexpectedly, how-

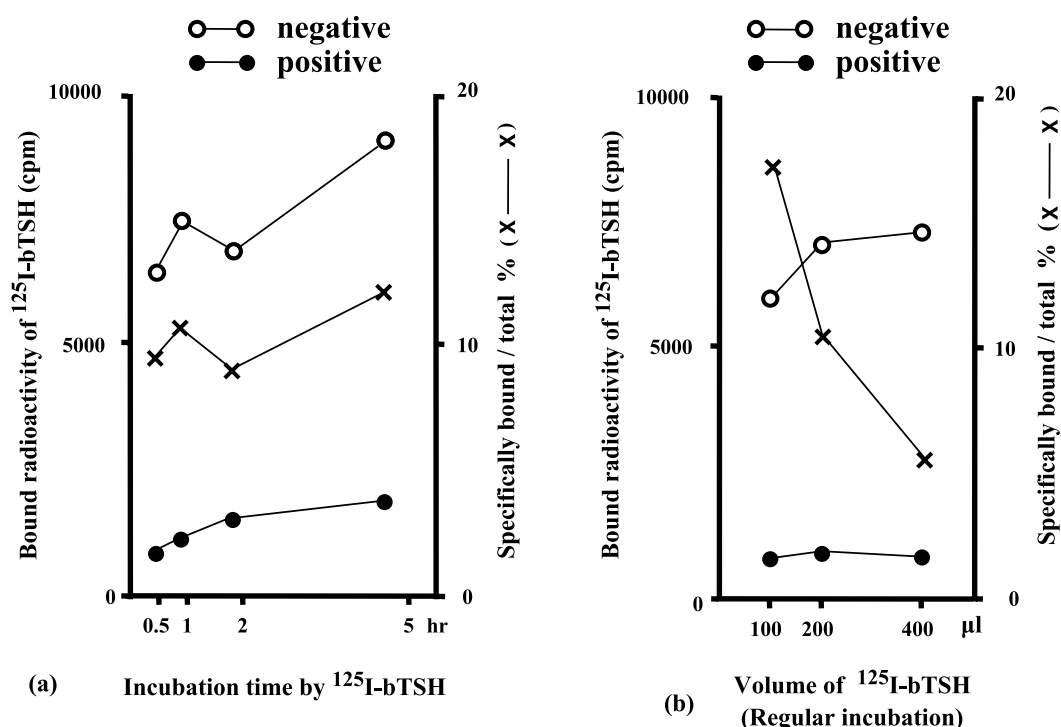


Fig. 2. Binding of 125 I-bTSH to the rhTSHR coated tube.

Using the negative and positive control substances provided, first incubation was performed for 2 hr. second incubation conditions with 125 I-bTSH were varied for time (0.5, 1, 2 and 5 hr) or applied volume (100, 200 and 400 μ l). Specifically bound/ total % was calculated as: (Cpm by negative control – Cpm by positive control)/ (Total cpm of 125 I-bTSH) \times 100 (%). Data of negative control, positive control and specifically bound % are presented by open circles, closed circles and \times marks, respectively. (a): Time-sequential effects. (b): Effects of dosage of 125 I-bTSH.

ever, alterations of second incubation time did not affect measured hTRAb activities at all. The results of 30 min incubation were almost equal to those of regular 1 hour incubation. Nor were decreases or even slight increases (though not significant) seen by extension of second incubation time. This was also the case in studies with different ^{125}I -bTSH doses (Fig. 3b). Applications of either inadequate (100 μl) or excess (400 μl) ^{125}I -bTSH did not affect hTRAb results at all. Bound cpm of ^{125}I -bTSH by negative control and positive control in these 2 experiments were similar to Fig. 2a and 2b. Bound cpm by patient's sera almost paralleled the alterations of calculated specifically bound cpm (data not shown).

4. Time-dependent changes in hTRAb activities measured by the present system

As shown in Fig. 1, when the first incubation time was extended overnight, measured hTRAb activities increased significantly. Time-sequential studies of the

first incubation were performed using 100 μl aliquots of the same 8 Graves' hTRAb positive sera. As shown in Fig. 4a, all measured hTRAb activities increased consistently and significantly with incubation time. Fig. 4b shows alterations of bound cpm of ^{125}I -bTSH. Gradual decreases in bound cpm with the first incubation time were seen by both negative and positive controls. However, specifically bound cpm was rather consistent up to 5 hours. On the other hand, bound cpm obtained by hTRAb samples showed apparent decreases with incubation time as seen by steep downward gradients. Quite different from the observations in ^{125}I -bTSH binding study, bindings of hTRAb to rhTSHR revealed gradual and cumulatively great increases depending on incubation time.

To further examine TSHR binding property of hTRAb and ^{125}I -bTSH, a 1 step incubation study was performed. Fig. 5a shows alterations of measured hTRAb activities in 50 μl aliquots of the same 8 Graves' sera obtained by 1 step incubation times of 1, 3, and 5 hr. The antibody activity measured by this

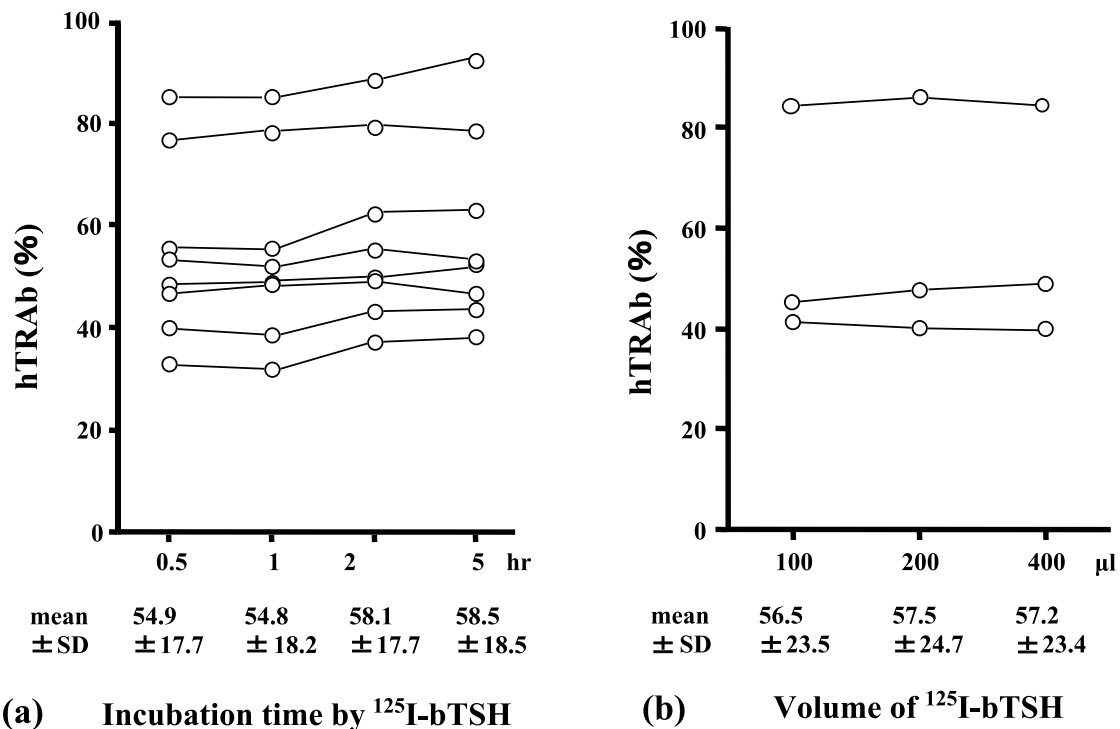


Fig. 3. Effects on hTRAb assay results of second incubation conditions with ^{125}I -bTSH.

(a): Effects of second incubation time. Using 50 μl aliquots of 8 known hTRAb positive Graves' sera, first incubation was performed for 2 hr. Then second incubation times with ^{125}I -bTSH were changed to 0.5, 1, 2 and 5 hr. Presentations of mean \pm SD are the same as in Fig. 1. (b): Effects of ^{125}I -bTSH dose applied. After 2 hr first incubation using 3 hTRAb positive sera, second incubations were performed with variable doses of ^{125}I -bTSH of 100, 200 and 400 μl , respectively. Presentations of mean \pm SD are the same as in Fig. 1.

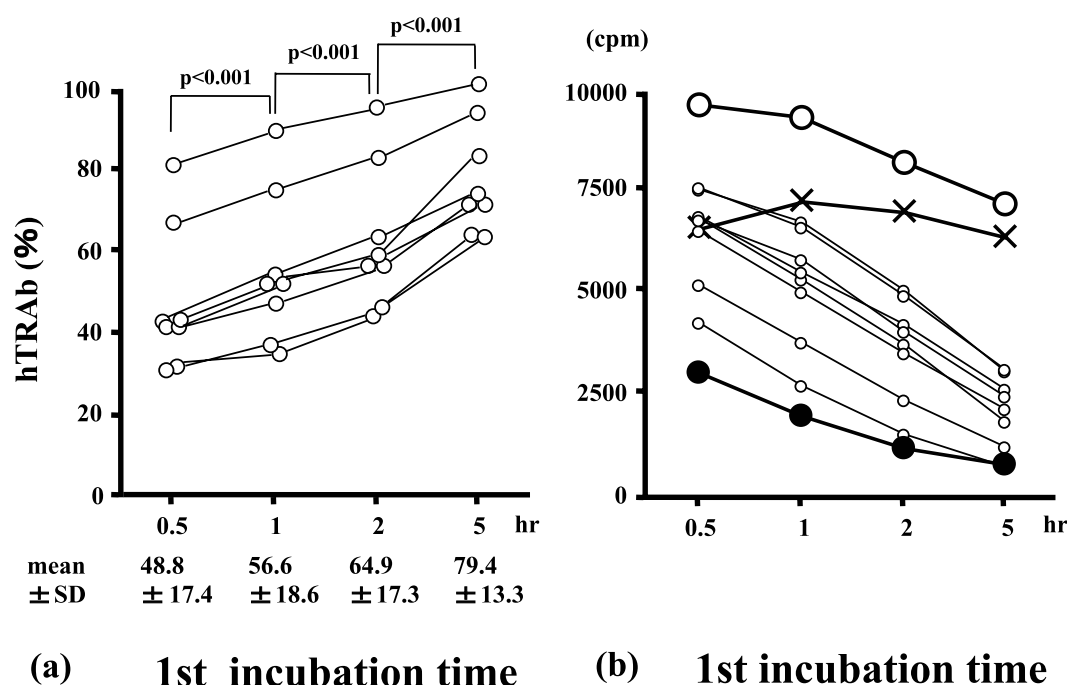


Fig. 4. hTRAb assay results in time sequential study of the first incubation.

Eight hTRAb samples were measured after various first incubation times from 0.5 to 5 hr. Second incubations were performed as usual. (a): hTRAb assay results. Presentations of mean \pm SD and statistical analyses are the same as in Fig. 1. (b): Bound cpm obtained by hTRAb sera, negative and positive controls, and the calculated specifically-bound cpm. Data of patients' sera, negative control, positive control and specifically bound cpm are presented by small circles, large open circles, closed circles and \times marks, respectively.

experiment appeared to be much closer to TBII than hTRAb, but we expressed this activity also as hTRAb, to avoid confusion. One hour incubation resulted in much lower hTRAb activities than the regular two step assay data by 50 μ l application ($p = 0.005$). At the same time a considerable part of hTRAb ($42.7\%/54.8\% = 77.9\%$) was shown to inhibit 125 I-bTSH binding even by 1 hour incubation. Of great interest, the hTRAb activities measured by the 1 step incubation revealed significant increases along with incubation time, and with 5 hr incubation the results were closer to the regular two step assay results. Compared to TBII activities, 1 hr results revealed even higher activities, though not significantly, and 3 and 5 hr results were significantly higher than TBII results. As shown in Fig. 5b, TSHR bound 125 I-bTSH cpm of negative control increased along with time, while that of positive control was almost stable, and calculated specifically bound cpm showed gradual increases along with time. In the cases of patients' sera, however, bound cpm increased significantly by 3 hours ($p = 0.002$), but decreased significantly by 5 hours ($p = 0.003$). TSHR binding of 125 I-bTSH was found to be inhibited gradu-

ally by co-incubation with hTRAb or partly displaced by hTRAb during simultaneous incubation.

Time sequence studies using conventional TBII assay system were then performed using 8 hTRAb positive sera, one of which was TBII negative. Preincubation time by serum sample was extended from regular 15 min to 120 min. As shown in Fig. 6, significant increases of TBII results were seen even by 30 min compared to regular 15 min results ($p < 0.001$). Further increases of TBII activities were observed until 120 min. Interestingly, the originally hTRAb positive but TBII negative sample turned out to be TBII positive by 60 min (12.0%) and 120 min (15.2%) incubation. In cases of 5 hTRAb and TBII negative sera, none of these showed positive results by prolonged preincubation (data not shown).

5. Time-dependent changes in thyroid stimulating activities by bTSH and Graves' IgG

Whether the delayed TSHR action observed by hTRAb and TBII can be applied to TSAb or not was then studied. As shown in Table 1, all but one of

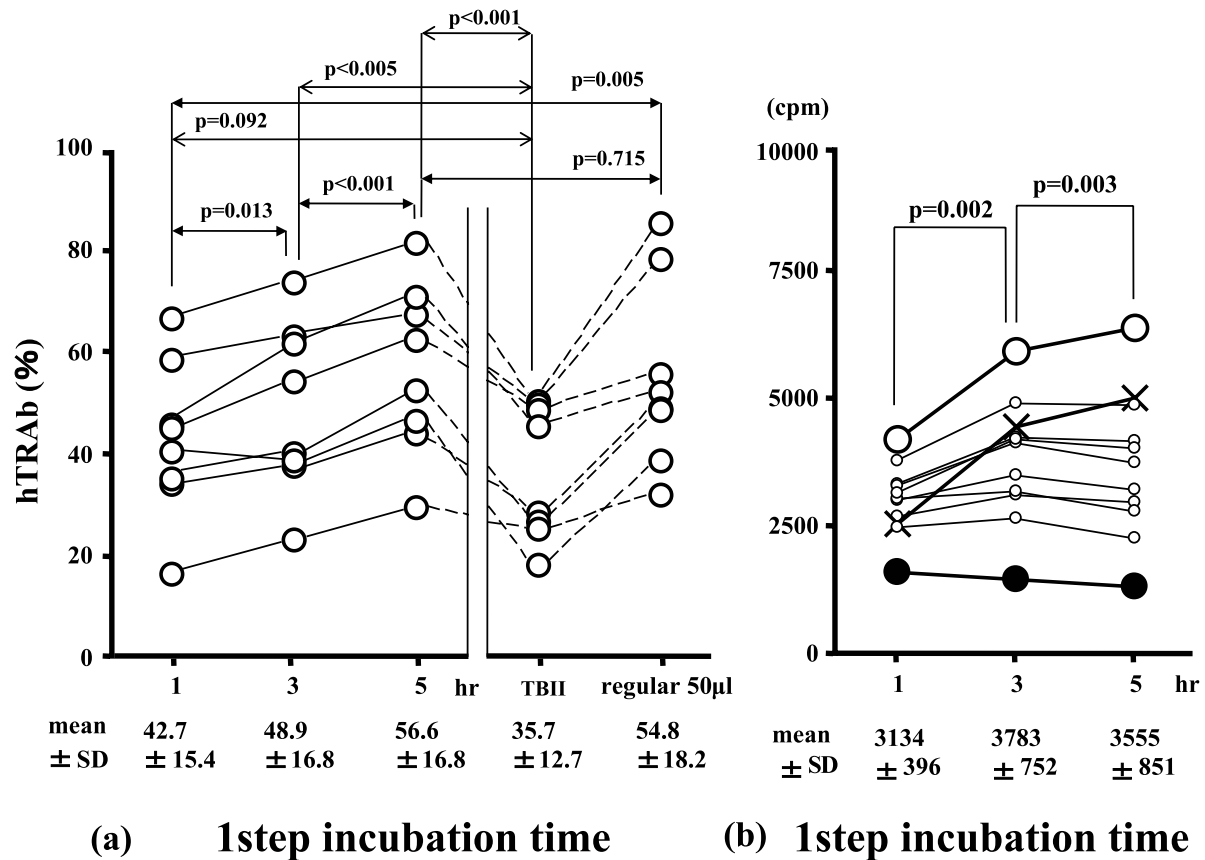


Fig. 5. Time-sequence hTRAb assay results obtained by the 1 step incubation study.

Fifty μ l aliquots of 8 TBII samples (those used in Fig. 3 and 5 experiments) with first incubation medium (200 μ l) and 125 I-bTSH solution (200 μ l) were applied simultaneously to rhTSHR coated tubes, and incubated for 1, 3 and 5 hours. Tubes were washed and counted for radioactivity. These experimental procedures were quite different from that of hTRAb assay, but to avoid confusion, we designated the results as hTRAb. (a): hTRAb assay results. As references, TBII assay results and regular hTRAb assay results using 50 μ l samples are shown. Presentations of mean \pm SD and statistical analyses are the same as in Fig. 1. (b): Bound cpm obtained by hTRAb sera, negative and positive controls, and calculated specifically bound cpm. Data of patients' sera, negative control, positive control and specifically bound cpm are presented by small circles, large open circles, closed circles and \times marks, respectively. Mean \pm SD of cpm obtained by patients sera are presented, and these are compared with each other.

thyroid stimulatory ligands exhibited positive TSAb activity with regular 4 hr incubation, while 1 Graves' IgG (Pt2) remained negative. As for bTSH, high dose of both T1 and T2 showed peak cAMP generation with 8 hr incubation and almost equal (T1) or decreased values (T2) were observed with 24 hr incubation. On the other hand, all 3 Graves' IgGs tested revealed further increases in cAMP generation by 24 hr than 8 hr incubation, and even Pt2 showed positive TSAb activity by 24 hr. Extension of incubation time was again shown to improve assay sensitivity. Another important finding was that the most potent TSAb sample (Pt3) generated much cAMP even by a short incubation, and that 75% (46.03/61.27) of cAMP was generated by 2 hr

incubation compared to regular 4 hr incubation. Compared to bTSH, Graves' IgG was found to show apparently delayed action to the TSHR not only in binding but also in *in vitro* thyroid stimulation.

Discussion

TSH and Graves' TBII have been considered to bind TSHR competitively, and therefore we measured TBII activity by grade of binding inhibition of TSH to the receptor [4, 6, 11]. A new sensitive TRAb assay system utilizes rhTSHR coated tubes and a two step incubation with patient serum incubated first and 125 I-bTSH sec-

ond [21, 22]. Using this system, we found apparent differences in the TSHR binding properties between TSH and Graves' hTRAb.

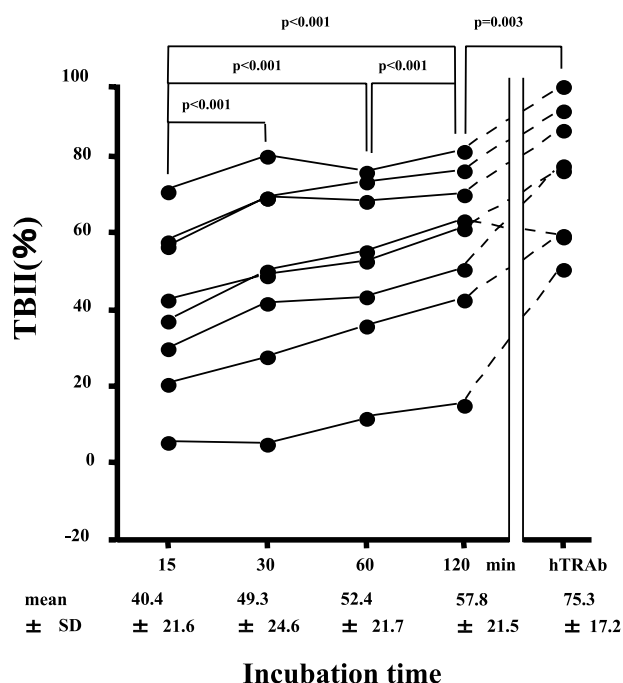


Fig. 6. Effects of incubation time on TBII activities measured by the conventional assay.

Using 8 hTRAb positive sera, conventional TBII assays were performed with various first incubation times from 15 min to 30, 60 and 120 min. second incubations were performed for 60 min as usual.

The rhTSHR coated tubes were shown to be almost completely saturated by 200 μ l of the 125 I-bTSH solution provided within 1 hr incubation. Extension of incubation time up to 5 hr or increase of applied 125 I-bTSH dose did not increase 125 I-bTSH binding. On the other hand, hTRAb assay results of Graves' sera increased greatly with overnight incubation. Since excessively long incubation with serum may cause receptor-damage, time sequence studies up to 5 hr were performed, which resulted in the finding that hTRAb increases depending on incubation time. As shown in Fig. 4b, specific binding of 125 I-bTSH decreased gradually depending on the length of the first incubation time. This may be the result of receptor damage occurring along with time. This was also the case for non-specific binding obtained by positive control serum. Subsequently, specifically TSHR bound cpm of 125 I-bTSH did not show any significant decrease up to 5 hr. Increases of hTRAb activity by incubation time could not be ascribed simply to the receptor damage by prolonged incubation, but were considered mostly due to increases of specific hTRAb binding to the TSHR. Quite similar phenomena were observed with time sequence study using conventional TBII assay system by the present study and previously by others as well [25, 26]. If hTRAb in an individual patient is a single moiety, such gradual increases in hTRAb results indicate that binding affinity of hTRAb should be much lower than that of TSH. On second incubation, 125 I-bTSH had been considered to bind TSHR competitively with

Table 1. Time-sequential effects on cAMP generation by cultured porcine thyrocytes by bTSH and TSAb from Graves' patients

Time	2 hr		4 hr		8 hr		24 hr	
Sample	pmol/mL	(%)	pmol/mL	(%)	pmol/mL	(%)	pmol/mL	(%)
Normal pool	3.50 (3.58, 3.41)	100	3.51 (3.67, 3.35)	100	3.27 (3.20, 3.34)	100	3.92 (3.87, 3.96)	100
T 1	12.38 (12.95, 11.81)	354	32.35 (32.48, 32.22)	922	47.34 (47.28, 47.40)	1448	47.16 (46.28, 48.03)	1203
T 2	10.04 (10.42, 9.66)	287	25.39 (26.12, 24.65)	723	37.94 (38.87, 37.01)	1160	28.81 (27.39, 30.22)	735
Pt1	4.83 (4.74, 4.91)	138	8.14 (8.08, 8.21)	232	11.51 (10.95, 12.06)	352	20.05 (18.88, 21.22)	511
Pt2	3.56 (3.45, 3.66)	102	4.71 (4.59, 4.83)	134	4.80 (4.42, 5.18)	147	7.05 (6.92, 7.18)	180
Pt3	46.03 (46.00, 46.06)	1315	61.27 (59.94, 62.60)	1746	70.10 (71.88, 68.32)	2144	91.48 (91.94, 91.01)	2333

Final concentration of 2 bTSH samples (T1 and T2) were adjusted to 400 μ IU/ml and 200 μ IU/ml, as described. IgG fractions were also prepared from 3 Graves' patients sera (Pt1, Pt2 and Pt3). Mean and duplicated cAMP assay results for each sample obtained by 4 incubation times are presented. TSAb activities at each incubation time were calculated as percent change of generated cAMP from that of normal IgG.

hTRAb [21, 22]. To our surprise, when the second incubation time and volume of ^{125}I -bTSH were altered, hTRAb assay results were not affected at all. The second incubation with ^{125}I -bTSH was found not to be a simple competitive binding but just a binding to the unoccupied TSHR.

In order to study the TSHR binding mechanisms of hTRAb and ^{125}I -bTSH further, we then performed a 1 step incubation which applied ^{125}I -bTSH and Graves' sera simultaneously. We first assumed that because of direct binding competition with ^{125}I -bTSH, hTRAb assay results should be decreased and could not be altered by extension of incubation time. Indeed, hTRAb results with 1 hr-incubation were lower than those with regular two step assay. However, quite unexpectedly, hTRAb results increased gradually and significantly in a time dependent manner up to 5 hr. ^{125}I -bTSH was not able to displace bound hTRAb at all, but not vice versa, since hTRAb could displace once-bound ^{125}I -bTSH during incubation. This indicates that reactions between 2 independent coincident binding materials with potentially different rebinding properties may prove to be very complicated and in need of further investigation.

We then studied whether similar prolonged TSHR actions can be seen in the case of TSAb as well. This was indeed the case, and comparing the time-course of cAMP generation by high doses of bTSH, Graves' TSAb of both potent and weak activities consistently revealed apparently delayed patterns. As is well known, Graves' IgG stimulates the thyroid of rodents in a much more delayed fashion than TSH [1, 2]. The mechanisms why Graves' IgG acts as LATS have been explained mostly due to the differences in metabolic turnover rate, much shorter for TSH than IgG. Through detailed structure-function analyses of the TSHR, Davies *et al.* [16] recently suggested that TSAb may induce receptor dimerization after binding, and that such dimers may get into the lipid raft slowly, while TSH forms monomers that can get into it rapidly. Our present data indicated differences in time sequential cAMP generations only. However, delayed *in vitro* TSHR actions by TSAb may also play a role in LATS action patterns.

Is TRAb in individual Graves' patients a single moiety or not? Autoantibodies, including TRAb have been considered to be polyclonal and subsequently heterogeneous [11, 21]. Watanabe *et al.* clearly showed the presence of different TRAb moieties among Graves'

patient [17]. Further, the presence of heterogeneous TSAb moieties in the epitope recognition in individual patient has also been shown [9, 18–20]. Our present observations that TRAbs bind and act TSHR gradually and time-dependently is better understood by accepting the presence of slow-acting components in individual TRAbs. On the other hand, considerable parts of hTRAb were also shown to inhibit TSHR binding of ^{125}I -bTSH by 1 step incubation within 1 hour under co-existence of ^{125}I -bTSH. This finding should be regarded as certain proof of the presence of rapid-acting components within individual hTRAb moieties. In the case of TSAb, a potent TSAb (Pt3) was also shown to induce considerable amounts of cAMP generation (75%) by 2 hr incubation compared to regular 4 hr incubation. Such early action cannot be entirely explained by the assumption described by Davies *et al.* [16], and indicates the presence of rapid-acting components even among individual TSAb moieties. From these considerations, we conclude that the second generation TRAb assay is able to detect slow-acting hTRAb, and this is one of the reasons for its increased sensitivity. Some slow-acting hTRAb may have low affinity to hTSHR and even lack biological activity, hence the detection of these may result in false positive results concerning clinical hyperthyroidism. High detection rates of hTRAb in patients with euthyroid Graves' ophthalmopathy, and positive hTRAb detections in some patients under clinical remission have been frequently reported [21, 22, 27]. Similar observations have also been reported in the case of TSAb assay, especially after the establishment of this sensitive assay [27–30].

We are now able to measure TBII, TSAb and TSBAb in patients' sera. The binding sites on TSHR of TSBAb have been known to be different from those of TSH and TSAb [9–11, 31, 32]. In patients' sera of active Graves' disease we detected both TBII and TSAb, but whether or not these two antibody activities are derived from one immunoglobulin molecule is a matter of great concern [33]. Through peripheral B lymphocyte cloning obtained from Graves' patients, we prepared quite a few TRAb-producing monoclonal lines, and found that each of these lines produced only one TRAb, whether TBII or TSAb, and never both [34, 35]. However, just recently 3 separate groups have reported successes in producing monoclonal antibodies (mAbs) which have both TSAb and TBII activities [36–38]. These 3 mAbs were shown to exhibit TSAb activity in the nanomolar range, compete variably with

TSH for TSHR binding, and recognize the α subunit of TSHR, the hormone-binding portion of the ectodomain [38]. These mAbs appear to mimic high affinity TRAb in Graves' sera very well. Effective substitutions of ^{125}I -bTSH by labeled mAb for TRAb assay have also been suggested [39, 40]. However, if labeled mAb cannot compete effectively against already bound hTRAb, which probably recognize different epitopes from that of mAbs, as was the case of ^{125}I -bTSH shown in this study, the substitution may not be superior to ^{125}I -bTSH. Whether usage of mAb can differentiate TSAb (or TBII) from TSBAb is another point of interest, but this also appears hard to work well because the second incubation with labeled mAb may not compete

with any of the bound hTRAb at all.

Finally, we concluded that none of TRAb assays now available commercially are of sufficient quality to fully meet clinical needs, and that we still need to work further to arrive at clinically useful assay methods.

Acknowledgments

The authors would like to express their sincere gratitude to Yamasa Corporation, Tokyo, Japan for providing numerous TRAb kits, and also for kindly performing the sequential measurement of TSAb activities.

References

- Adams DD, Purves HD (1956) Abnormal responses in the assay of thyrotropin. *Proc Univ Otago Med School* 34: 11–12.
- McKenzie JM (1958) The bioassay of thyrotropin in serum. *Endocrinology* 63: 372–382.
- Kriss JP, Pleshakov V, Chien JR (1964) Isolation and identification of the long-acting thyroid stimulator and its relation to hypothyroidism and circumscribed pretibial myxedema. *J Clin Endocrinol Metab* 24: 1005–1028.
- Shering G, Smith BR (1982) An improved radioreceptor assay for TSH receptor antibodies. *Clin Endocrinol (Oxf)* 17: 409–414.
- Orgiazzi J, Williams DE, Chopra IJ, Solomon DH (1976) Human thyroid adenylate cyclase stimulating activity in immunoglobulin G of 341 patients with Graves' disease. *J Clin Endocrinol Metab* 42: 341–354.
- Smith BR, McLachlan SM, Furmaniak J (1988) Autoantibodies to the thyrotropin receptor. *Endocr Rev* 9: 106–121.
- Parmentier M, Libert F, Maenhaut C, Lefort A, Gerard C, Perret J, Sande JV, Dumont JE, Vassart G (1989) Molecular cloning of the thyrotropin receptor. *Science* 246: 1620–1622.
- Nagayama Y, Kaufman KD, Seto P, Rapaport B (1989) Molecular cloning, sequence and function expression of the cDNA for the human TSH receptor. *Biochem Biophys Res Commun* 165: 1184–1190.
- Mori T, Akamizu T, Kosugi S, Sugawa H, Inoue D, Okuda J, Ueda Y (1994) Recent progress in TSH receptor studies with a new concept of autoimmune TSH receptor disease. *Endocr J* 41: 1–11.
- Kohn LD, Shimura H, Shimura Y, Hidaka A, Giuliani C, Napolitano G, Ohmori M, Laglia G, Saji M (1995) The thyrotropin receptor. *Vitam Horm* 50: 287–384.
- Rapaport B, Chazenbalk GD, Jaume JC, McLachlan SM (1998) The thyrotropin (TSH) receptor: interaction with TSH and autoantibodies. *Endocr Rev* 19: 673–716.
- Szkudlinski MW, Fremont V, Ronin C, Weintraub BD (2001) Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships. *Physiol Rev* 82: 473–502.
- Chazenbalk GD, Pichurin P, Chen C-R, Latrofa F, Johnstone AP, McLachlan SM, Rapaport B (2002) Thyroid-stimulating autoantibodies in Graves' disease preferentially recognize the free α subunit, not the thyrotropin receptor. *J Clin Invest* 110: 209–217.
- Latif R, Graves P, Davies TF (2001) Oligomerization of the human TSH receptor reveals post-translational complexes. *J Biol Chem* 276: 45217–45224.
- Jeffereys J, Depraetere H, Sanders J, Oda Y, Evans M, Kiddie A, Richards T, Furmaniak J, Smith BR (2002) Characterization of the thyrotropin binding pocket. *Thyroid* 12: 1051–1061.
- Davies T, Marians R, Latif R (2002) The TSH receptor reveals itself. *J Clin Invest* 110: 161–164.
- Watanabe Y, Tahara K, Hirai A, Tada H, Kohn LD, Amino N (1997) Subtypes of anti-TSH receptor antibodies classified by various assays using CHO cells expressing wild-type, or chimeric human TSH receptor. *Thyroid* 7: 13–19.
- Kim WB, Chung HK, Lee HK, Kohn LD, Tahara K, Cho B-Y (1997) Changes in epitopes for thyroid-stimulating antibodies in Graves' disease sera during treatment of hyperthyroidism: therapeutic implications. *J Clin Endocrinol Metab* 82: 1953–1959.
- Ueda Y, Sugawa H, Akamizu T, Okuda J, Ueda M, Kosugi S, Ohta C, Kihou Y, Mori T (1995) Thyroid-

- stimulating antibodies in sera from patients with Graves' disease are heterogeneous in epitope recognition. *Eur J Endocrinol* 132: 62–68.
20. Sugawa H, Akamizu T, Kosugi S, Ueda Y, Ohta C, Okuda J, Mori T (1995) Presence of heterogeneous thyroid-stimulating antibodies in sera from individual Graves' patients as shown by synthesized thyrotropin receptor peptide application: evidence showing two independent epitopes and a possible recognition of two epitopic regions by one antibody molecule. *Eur J Endocrinol* 133: 283–293.
 21. Costagliola S, Morgenthaler NG, Hoermann R, Badenhop K, Struck K, Freitag D, Poetl S, Weglohner W, Hollidt JM, Quadebeck B, Dumont JE, Schumm-Draeger P-M, Bergmann A, Mann K, Vassart G, Usadel K-H (1999) Second generation assay for thyrotropin receptor antibodies has superior diagnostic sensitivity for Graves' disease. *J Clin Endocrinol Metab* 84: 90–97.
 22. Morgenthaler NG (1999) New assay systems for thyrotropin receptor antibodies. *Thyroid* 6: 251–260.
 23. Zweig MH, Campbell G (1993) Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* 39: 561–577.
 24. Inui T, Kouki T, Yamashiro K, Hachiya T, Ochi Y, Kajita Y, Sato Y, Nagata A (1998) Increase of thyroid stimulating activity in Graves' immunoglobulin-G by high polyethylene glycol concentrations using porcine thyroid cell assay. *Thyroid* 8: 319–325.
 25. Kuroyanagi M, Ishikawa K, Watanabe Y (2001) Studies on the improvement of assay sensitivity of TRAb measurement. Presented at the 44th annual meeting of Japan Thyroid Association. Naha, Japan.
 26. Kamijo K (2003) TSH-receptor antibody measurement in patients with various thyrotoxicosis and Hashimoto's thyroiditis: a comparison of two-step assays, coated plate ELISA using porcine TSH-receptor and coated tube radioassay using human recombinant TSH-receptor. *Endocr J* 50: 113–116.
 27. Kasagi K, Misaki T, Konishi J (2002) A sensitive solid-phase assay for TSH binding inhibitor immunoglobulins using recombinant human TSH receptor. *Jpn J Med Pharm Sci* 47: 305–310 (in Japanese).
 28. Mori T, Orgiazzi J (1995) TRAbs, When to measure? Presented at the 11th International Thyroid Congress, Toronto, Canada.
 29. Kasagi K, Konishi J, Iida Y, Ikekubo K, Mori T, Kuma K, Torizuka K (1982) A new in vitro assay for thyroid stimulator using cultured thyroid cells: effect of sodium chloride on adenosine 3', 5'-monophosphate increase. *J Clin Endocrinol Metab* 54: 108–114.
 30. Kosugi S, Mori T, Imura H (1989) Mechanisms by which low salt condition increases sensitivity of thyroid stimulating antibody assay. *Endocrinology* 125: 410–417.
 31. Tahara K, Ban T, Minegishi T, Kohn LD (1991) Immunoglobulins from Graves' disease patients interact with different sites on TSH receptor/LH-CG receptor chimeras than either TSH or immunoglobulins from idiopathic myxedema patients. *Biochem Biophys Res Commun* 179: 70–77.
 32. Kosugi S, Ban T, Akamizu T, Kohn LD (1992) Identification of separate determinants on the thyrotropin receptor reactive with Graves' thyroid-stimulating antibodies and with thyroid-stimulating-blocking antibodies in myxedema: these determinants have no homologous sequence on gonadotropin receptors. *Mol Endocrinol* 6: 168–180.
 33. McLachlan SM, Rapaport B (1996) Editorial: monoclonal, human autoantibodies to the TSH receptor — The holy grail and why are we looking for it? *J Clin Endocrinol Metab* 81: 3152–3154.
 34. Sugawa H, Akamizu T, Inoue D, Okuda J, Mori T (1993) Production of clonal anti-thyroid stimulating hormone receptor antibody producing B cell lines using Epstein-Barr virus transformation. In: Nagataki S, Mori T, Torizuka K (eds) 80 years of Hashimoto disease. Excerpta Medica, Amsterdam, 491–495.
 35. Akamizu T, Matsuda F, Okuda J, Li H, Kanda H, Watanabe T, Honjo T, Mori T (1996) Molecular analysis of stimulatory anti-thyrotropin receptor antibodies (TSAbs) involved in Graves' disease. *J Immunol* 157: 3148–3152.
 36. Sanders J, Jeffreys J, Depraetere H, Richards T, Evans M, Kiddie A, Brereton K, Groenen M, Oda Y, Rees Smith B (2002) Thyroid stimulating monoclonal antibodies. *Thyroid* 12: 1043–1050.
 37. Ando T, Latif R, Pritsker A, Moran T, Nagayama Y, Davies TF (2002) A monoclonal thyroid stimulating antibody. *J Clin Invest* 110: 1667–1674.
 38. Costagliola S, Franssen JD, Bonomi M, Urizar E, Willnich M, Bergmann A, Vassart G (2002) Generation of a mouse monoclonal TSH receptor antibody with stimulating activity. *Biochem Biophys Res Commun* 299: 891–896.
 39. Costagliola S, Vassart G (2002) Monoclonal antibodies with thyroid stimulating activity, at last. *Thyroid* 12: 1039–1041.
 40. Morgenthaler NG (2003) Clinical usefulness of the TRAK human assay. Presented at the 76th annual meeting of Japan Endocrine Society. Yokohama, Japan.